

12 August 1982

Disunited nations in outer space

The UN conference on space technology opened in Vienna earlier this week is certain not to ask the questions that need answering. So should conferences like these take place at all?

The United Nations conference on the peaceful uses of outer space, which opened this week in Vienna, seems to have set off on a familiar track (see page 595). It is right and proper, but also predictable, that the Austrian Foreign Minister, Dr Willibald Pahr, should have opened the proceedings with a declaration that the most urgent need is somehow to rid what the United Nations calls outer space of all military connotations. Better this, it may be thought, than that the keynote address should be yet another plea that the benefits of remote sensing satellites (whatever they may be) should be made freely accessible to all sovereign states that think they have a need of them, or which are just curious to know what they look like from aloft. Certainly it is far better than that the members of the group of seventy-seven, the developing countries whose interests in development are a part but only a small part (witness present anxieties about the Lebanon) of the proper objectives of the United Nations, should set up a howl that they do not have the direct-broadcasting satellites to put into the geosynchronous orbits with which the United Nations (through the International Telecommunications Union) endowed them in 1979. Even so, it is a serious question for the United Nations to consider whether conferences like these should continue to be held.

None of this is to suggest that the military use of space technology is not a serious problem. The past few years have shown all too well that the contrary is the truth. The major powers, principally the United States and the Soviet Union, are already dependent on satellites for much of their military intelligence. Quite soon there may be systems for putting other systems out of action (see *Nature* 15 July, p.211). And the process seems destined to catch on. Other states (France, for example) will soon be up there too. Other systems, perhaps laser weapons, will be added to the orbital ironmongery. Moreover, there seems no doubt that once these systems have multiplied the danger that they will be used provocatively if not in anger will be substantial. The trouble, unfortunately, is that no amount of speech-making at United Nations conferences will bring the perpetrators of these new devices to an agreement on restraint. Only hard-headed negotiations between parties willing that negotiations should take place could arrive at such a conclusion. Some international organization could, it is true, help to bring those who now use orbits for military (but not destructive) purposes to heel by keeping a close watch on what they are about, and then publishing the results. This is what governments such as that of Sweden have been urging. Unfortunately, for the United Nations to act in such a sense, either the political backing of the major powers or the financial backing of the others would be necessary. Neither is likely to be forthcoming.

So what, in these circumstances, can the latest conference hope to accomplish? By the end of next week, the delegations will no doubt be hard at work on the draft of a declaration so stuffed with anodyne pieties that even hard-headed governments will be shamed into endorsement. One certain ingredient in the communicate — would that this prediction were proved wrong — will be that the benefits of telecommunications by satellite, the only commercially proven benefit of these devices, should be more freely available and more widely shared. Ever since the disastrous United Nations Conference on Science, Technology and Development (also at Vienna) in 1979, the notion has become

entrenched that it is somehow inequitable that high technology should be exploited first by highly developed industrialized states. But this clamour is entirely mistaken. If industrialized states are to be persuaded not to win some commercial benefit from their high technology, how can they be asked (as they should be asked) to throw open their domestic markets more freely to imports from the developing countries?

This contradiction lies at the heart of much of what the United Nations seeks to accomplish in these periodic and increasingly gigantic conferences. Their form ensures that all parties go away frustrated — the high-technology states because they have been shamed into promises whose only saving grace is that they are empty, the others because they believe (or pretend) that the promises mean what the words say. The outcome, mutual despair, may be as dangerous as the militarization of space over which Dr Pahr wrung his hands this week. Has the time come when somebody should say no to the next conference (on nuclear energy next year) or at least to the one after that?

Gamekeeper turned poacher

The British need a new university boss; herewith some nominations and a job description.

Dr Edward Parkes seems a glutton for punishment. Most of his spell as chairman of the University Grants Committee has been spent in forcing arbitrary government economies on British universities. His decision now to quit is understandable, and would have been forgiven at any time in the past few years. His decision to be instead the next vice-chancellor of the University of Leeds is more surprising. Is it self-sacrificing heroism, a wish to be seen lying on the bed of nails that universities accuse him of having fashioned for them? Or bravado, an ambition to show that the economies are not so bad after all? Either way, the University of Leeds, already distinguished among British universities not merely for its academic strength but, these days even more important, for the strong regional loyalty that sustains it, must count itself lucky. For a university to have as its chief officer someone who knows how the grants committee functions must be an advantage of some kind. Parkes is unlikely to use his position to win extra leverage for Leeds with the grants committee, although many of his new colleagues are hoping that he is not that straight. But at the worst, he will be able to help his new university to understand more readily than others what may lie behind the inscrutable instructions that issue from time to time from the committee he is now leaving.

For the British government, Parkes's departure now is probably more of an embarrassment than an opportunity to fill the empty office with a kind of stool-pigeon or Uncle Tom. Not that there is a shortage of potential successors to Parkes. Sir Alec Merrison, vice-chancellor at Bristol, would suit quite well, even if Sir Peter Swinnerton-Dyer from Cambridge would be more interesting. Lord Flowers, rector of Imperial College London, would be a strong appointment, but would the government welcome a grants committee chairman free to speak his mind in the House of Lords? Lord Swann, under-employed since quitting as head of Oriel College, Oxford, a year ago, is similarly



encumbered by a peerage. But then Sir Ronald Mason will be leaving his post as scientific adviser to the Ministry of Defence later in the year, while Mr Geoffrey Caston, who has administered the vice-chancellors' own lobby (the Committee of Vice-Chancellors and Principals) for the past five years, probably knows as intimately as anybody how the British university system functions. Any one of these, and no doubt a host of others, could embark on a stint as chairman of the grants committee without having first to win the confidence of British universities. The government would even find that most of them accept its own view that even when direct support for the universities has levelled off in 1984-85, continuing reorganization will be necessary within the British system. But none of them would take Parkes's job without certain assurances, the most important of which is that there should be an understanding between the government and the universities on what the grants committee is for.

What should they ask for? The natural temptation is to ask for more independence, but that will not be as simple as it seems. Although the grants committee was established to be an independent buffer between the universities and the government, it has never been autonomous. In principle, in the old days, the government would say what subsidy it could afford, the committee would recommend how that should be divided and the government would send out cheques to individual universities accordingly. In practice, of course, the grants committee was very much a spokesman for the university system, arguing the case for new developments in some field or other (more medicine, more science perhaps), whose credibility with the government stemmed from its periodic invigilation of how universities individually conducted their affairs. So even in the halcyon days when the committee dealt with the Treasury rather than with the Department of Education and Science, and when the scale of spending on the universities was settled five years ahead, the government was inevitably drawn into decisions about the character as well as the scale of British higher education. Most of the changes that have come about in the past few years are changes only of degree. After the government decreed at the end of 1980 that its subsidies for the universities should be reduced by 8.5 per cent in real terms, it seems genuinely to have been left to the grants committee to decide how the misery should be distributed. The declaration a few weeks ago by the Secretary of State for Education and Science that ministers might in future shoulder more responsibility for issues of university policy is therefore not an innovation of principle. The danger is merely that they will choose to intervene too often and too trivially.

Candidates for Parkes's job will therefore ask for an assurance that they will have a job to do, and not be mere office-boys (or girls?) for ministers. More important, they should also ask for a restoration of the old civilities by which the grants committee was consulted in advance on important issues of public policy on higher education. Parkes appears to have been told in advance of the two decisions that have most affected British universities in the past few years, the increase of fees for students from overseas and the reduction of the general subsidy (coupled with limits on student numbers). The government may now regret that on either occasion was the grants committee given a chance to suggest how its objectives might be more sensibly, and more equitably, attained. Yet even these examples of the present government's disrespect for the grants committee (to some extent made good by Sir Keith Joseph's offer of a "dialogue") are not unprecedented. The creation in the 1960s of what is called the "binary" system of higher education, with parallel systems of universities and polytechnics, was presented to the universities by the late Mr Anthony Crosland as a bolt from the blue. Yet that has become the most serious unsolved problem in British higher education. In the government's interest as well as his own, Parkes's successor should make it clear in advance that if he first learns of radical changes in his working environment from the morning newspapers, he will be up and off before the day is out.

Given suitable assurances, Parkes's successor could have a rewarding job. In the promised period of stability after the present contraction is over, it should be possible to make a start on

the restoration of the spirits of academics and of their universities. The new grants committee chairman will have the advantage of dealing with universities that, by definition, will have survived, sometimes to their own surprise. Many of them will by then be enlivened by innovations forced out of them in these hard times, from science parks and essays in continuing education to the discovery that research of high quality does not necessarily require an entail on the output of a gold mine. Administratively, so far as the universities are concerned, the trick must be to find a way of giving all universities a sense that they are more fully than at present in control of their own destinies, from which would follow the beneficent consequence that they have an incentive to be different from each other, not an incentive to ape each other — or Oxbridge. (The criteria of academic standards and the conventions of "peer review" might have to be subordinated to others, the interests of students perhaps.) Politically, the objective would be to provide the government with a solution to the problem of the binary system, showing how it might be made unitary. Philosophically the need is for a chairman who could help to redefine the functions of higher education in countries such as Britain now that the participation rate is high enough to falsify the traditional academic assumption that the purpose of being a student is to become an academic. Such a chairman might even be able to find a way of creating some tangible linkage between higher education and national prosperity — everybody's ambition and nobody's boast. Then, so the daydream goes, British universities might have friends again in the House of Commons . . . Is that too much to hope for?

Jumpiness of bankers

Science and technology, usually respectful of banking, will suffer if bankers lose their nerve.

Why are the world's bankers so much on edge? In the past few weeks, these ordinarily imperturbable figures have taken to whispering behind their hands as if they were selling not credit (a respected commodity) but something disreputable. The change of demeanour is in the short term easily explicable. The bankers worry that they may not be permitted by their governments to roll over their credits to Poland when that question has to be decided next month, or that some unsophisticated member of their community may not agree to forgo payment on its Polish loan even if the governments concerned suggest that bankers should be compliant. But that is very much a short-term calculation. The sum of what the Polish government owes the Western banks, estimated at \$15,000 million or thereabouts, is perhaps only 5 per cent of what the commercial banks of the industrialized West have lent overseas in the past few years. Obviously, a default by Poland would be an inconvenience, but arithmetically it should not spell the end of the Western way of life. Western bankers will not be readily forgiven if they let the Polish credits jeopardize their survival — and that of their depositors.

So what really makes them jumpy? A sense of guilt, the certain knowledge that they are no longer as much in charge of their own affairs as they pretend they are. Once upon a time, when bankers earned their reputation for probity, banks undertook to look after the money entrusted to them by depositors, lent that to borrowers who had a need of it, and made their living on the turn. For at least half a century, however, the banks have been partly the prisoners of their governments — or suckers for their governments' propaganda. In the 1960s, the banks helped pay for the Vietnam war by exporting the US government's excess expenditure through the Eurodollar market, exporting inflation at the same time. More recently, in countries such as Britain and the United States, the banks have helped their governments both fiscally (with high interest rates that cause deflation) and politically (by lending to unknown clients overseas). Their profit margins have been assured, but their assets have been just as much at risk as are other people's. Discrimination, the banker's stock in trade, has gone by the board. Who, except the rest of us, will cry if they go bust?

More trouble for Stanford's patent

US patent office gives reasons for refusal

Washington

Further doubt has been cast on the validity of the key Cohen-Boyer patent covering basic processes in genetic manipulation by a decision on a second patent application taken last week by the US Patent and Trademark Office. On 30 June, the office announced that the granting of the second patent, originally planned for 13 July, would be postponed (see *Nature* 29 July, p.409). Last week, the office took a further step in the minuet-like US patent process and notified Stanford University (acting on its own behalf and that of the University of California, San Francisco) that the second patent application was to be rejected.

The decision of the patent office seems to turn on the status and origin of the plasmid called pSC101, that first used by Cohen and Boyer as a means of cloning foreign genes in *Escherichia coli* plasmids. Originally, Cohen and Boyer considered that this plasmid had been derived from a naturally occurring plasmid called R6-5 by recircularization of some fragment itself produced by hydrodynamic shear.

Last week, the US patent office cited an article published by co-inventor Stanley Cohen and a collaborator in 1977 (Cohen, S.N. and Chang, A.C.Y. *J. Bact.* 132, 734; 1977) in which other possible origins of pSC101 were canvassed. The possibility of a relationship with the *Salmonella* plasmid SP219 was mentioned.

The patent office now says that the 1977 article "sets forth a revised view of the origin of the pSC101 plasmid. That view conflicts with the origin of pSC101 as set forth in this application".

The first patent, which was issued to Stanford and the University of California at San Francisco in December 1980, presumably suffers from the same potential defect. The patent office has the authority to reexamine an already-issued patent, but has only exercised this option once since it was given the authority in 1980. Alternatively, a third party could file a challenge to the first patent.

A possible counter to the patent office's objection would be to show that the plasmid was publicly available at the time of application, and thus the claims in the patent for how the plasmid is used as a starting point in the creation of recombinant plasmids does not depend on the accuracy of the recipe for making pSC101. This matter is open to question; Cohen and his co-worker Herbert Boyer did make the plasmid available to qualified

scientists who requested it, but with some restrictions. The patent office asked Stanford to provide data on how many scientists were supplied the plasmid and whether any were refused.

Cohen said last week that the 1977 *Journal of Bacteriology* article "did not admit an error in the original procedure" but rather only in the "interpretation" of where the pSC101 plasmid came from. He

said that procedure was totally reproducible.

Stanford has already sold one-year licences to the first patent to 73 companies. Stanford has three months to respond to the patent office's action; a Stanford official said he did not see any "insurmountable obstacles" in successfully asserting its claim.

Stephen Budiansky

Space wars at UN conference

Vienna

Austrian Foreign Minister Willibald Pahr has called on the world scientific community to "oppose vigorously" the increased militarization of space. He was addressing the opening session of Unispace 82, the second United Nations conference on the exploitation and peaceful uses of outer space, now meeting here. Dr Pahr had just been elected president of the conference, an honour shared with Dr Kurt Waldheim, who, in 1968, had been Austrian Foreign Secretary when the first Unispace conference was held in Vienna.

Unispace-1 had been mainly concerned with the potential of the new space technologies. Unispace-2, fifteen years later, is interested primarily in what can be done with those technologies to improve the quality of life of mankind. An exhibition, staged parallel to the conference, shows an impressive range of space technologies.

In one respect, Unispace-2 might be considered as a showcase for the space techniques of the more developed nations, in their hopes that these benefits could be extended to the less fortunate.

UK Minister of State Kenneth Baker observed that, "Unispace is particularly significant for those countries which do not have their own space industry, but who seek quite rightly the assurance that the way that space applications are developing is of value to all".

The prestige element was, of course, present. The Soviet exhibit was under the direction of Dr A. P. Kapitsa, son of the Nobel prizewinner. The Americans brought to their stand not only their shuttle astronaut Henry Hartsfield but also a female trainee astronaut. The American impact, however, was somewhat lessened by the recent Soviet media campaign which stressed the military aspects of the shuttle and by fears of what President Reagan's 4 July speech about a "more permanent" US presence in space might mean — a subject on which US delegates to Unispace 82 were remarkably non-communicative.

The idea that the "demilitarization" of space was a prime prerequisite for its peaceful exploitation was taken up by many of the delegates, including the representatives of non-governmental

organizations, who have a special session on this theme scheduled for Saturday. If, unlike its predecessor, Unispace-2 produces some concrete recommendations, the peace issue is almost certain to be among them. So far, however, no concrete proposals for the "demilitarization" of space have emerged.

Vera Rich

India's space hopes

Bangalore

India's ambitions in space remain high despite a number of setbacks. Next month, the indigenous rocket, SLV-3, will carry a 42-kg satellite during its first operational flight and next year the first Indian astronaut will fly aboard the Soviet spacecraft Salyut-7. But the country's most ambitious space project yet, the multi-purpose operational domestic satellite INSAT-1A, has been crippled by the failure of its solar sail to deploy.

INSAT-1A, the world's first civilian telecommunications, weather and direct broadcasting satellite rolled into one, was built by the Ford Aerospace Corporation under contract from the Indian Space Department and launched by the US National Aeronautics and Space Administration aboard a Thor Delta rocket. The Indian government had planned to use the satellite for meteorology, long distance communications and for broadcasting direct to remote villages, but the failure of the solar sail will reduce the satellite's lifespan to two and a half years and limit its usefulness. The Indian Space Department is hoping for better luck with INSAT-2, due for launch aboard the space shuttle in July 1983.

High hopes are also pinned on the operational launch of SLV-3, India's own solid-fuelled, four-stage rocket. After a disastrous first test flight in 1979, SLV-3 had a successful test flight in July 1980 and was partially successful in May 1981 when it placed a small satellite into an unexpectedly low orbit, thereby condemning the satellite to 9 rather than 90 days of life.

B. Radhakrishna Rao

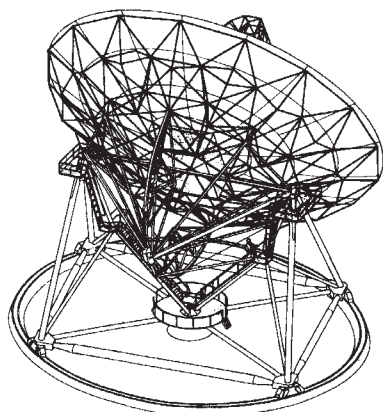
US astronomy

Arrays in, dishes out

Washington

The US radioastronomy community, after much agonizing, has decided to give first priority to the construction of the Very Long Baseline Array (VLBA) radio telescope — a decision that effectively means that the 25 metre telescope — which it has sought for several years without obtaining funds — will not now be built.

Associated Universities Inc., the management group that runs most major US radioastronomy facilities, recently submitted a formal proposal to the government asking for \$51 million in 1982



The 25-m millimetre wavelength telescope

dollars to build the network, which would extend from Alaska to Hawaii and across the continental United States to New England.

Using interferometric techniques, VLBA will have a baseline on the order of the radius of the Earth, and so will be able to achieve extraordinary resolutions of 0.3 milliarcsecond. Thus it will be able to study many aspects of the so-called "violent universe", such as explosive events in quasars and in the nuclei of galaxies. It may also permit precise distance determinations to maser sources in our own galactic system."

The 25-metre telescope, on the other hand, would have used very short, millimetre wavelengths to investigate interstellar molecular chemistry. It was first recommended in 1972 by a committee of the National Academy of Sciences (NAS) headed by Jesse L. Greenstein of the California Institute of Technology. The National Radio Astronomy Observatory in Charlottesville, Virginia, has sought funds for the 25-metre telescope since 1977; in some of those years, the National Science Foundation forwarded the request to the White House, where, for unrelated budgetary reasons, it stopped. Meanwhile, related work has continued at smaller dishes at Caltech, the University of California at Berkeley, Bell Laboratories and the University of Massachusetts.

Other countries, too, entered the promising field: the United Kingdom has a 15-metre dish at Mauna Kea, in Hawaii,

while the Franco-German telescope institute IRAM (Institut de la Radioastronomie Millimétrique), based at Grenoble in France, plan two telescopes — one almost ready at Pico Veleta in the Sierra Nevada near Granada in Spain, the other under construction at the Plateau de Bure in the southern French Alps (near Grenoble). The Japanese, too, plan to build one, and a copy of the dish destined for Granada has even been ordered by the government of Iraq.

But in spite of its once-widespread support in the United States, "time has passed the 25-metre by", as one astronomer said last week. The most recent review of astronomical priorities, a report by another NAS committee headed by Harvard astronomer George Field (see *Nature* 8 April, p.482), included a subcommittee to set priorities in radio astronomy hardware, headed by Pat Thaddeus of Columbia University's Institute for Space Studies. At that time, the 25-metre telescope seemed likely to be funded, so the final Field report gave top priority to the need for VLBA. Only afterwards, was it clear that even in fiscal year 1983, the 25-metre telescope would not be funded. So its virtual omission from the Field report meant it was doomed.

US radioastronomers hold mixed views about this gap in the field, in which the United States was a pioneer.

On the other hand, nobody disputes that VLBA, if it is built, will be a major advance, as nothing like it is planned elsewhere in the world.

Deborah Shapley

Fields Medal awards

Two Americans and a Frenchman are the 1982 winners of the Fields Medal, the "Nobel Prize" of mathematics, it was announced in Warsaw on Sunday.

The International Congress of Mathematicians (ICM), meeting this year in Poland, awarded the medal to William Thurston of Princeton University, S. T. Yau of the Princeton Institute of Advanced Studies, and Alain Connes, who is at present a director of research at the headquarters of the Centre National de la Recherche Scientifique in Paris. As a mathematician, Connes is attached to the Ecole Normale Supérieure and the University of Paris VI.

The Fields Medal, an inspiration of an early president of ICM, is awarded every four years. These are the ninth and tenth awards for Americans, and the sixth for a Frenchman — a remarkable record for France, bearing in mind that the United States has four times the population of France.

Robert Walgate

British academic pay

Five per cent

Last Wednesday a council meeting of the Association of University Teachers (AUT) approved a 5 per cent pay increase for university lecturers. It had little choice. When talks between AUT and the Universities Authorities Panel (the body nominated by the Committee of Vice-Chancellors and Principals to negotiate salaries) reached an impasse, the matter went to arbitration and 5 per cent was recommended. The award, which has been approved by Sir Keith Joseph, Secretary of State for Education, means that the universities will be faced with yet another book-balancing feat. The outcome may be further job losses.

Negotiations between AUT and the vice-chancellors were mainly over how much the universities could afford to set aside for salaries. Initially the vice-chancellors said they could afford nothing. The body both sides most wanted to negotiate with was the government. Each made representations to the government for more money to meet a pay increase. But the government remained adamant that any award must be met from the grants given to the universities by the University Grants Committee. The government did decide, however, to provide £770,000 to meet two-thirds of the cost of the 6 per cent award made to clinical academics to maintain their parity with National Health Service workers. "That is in some way understandable", commented Mr Laurie Sapper, general secretary of AUT, "After all, most government ministers are over 50 and don't want to upset the medics".

AUT was hoping for a much higher award to keep in line with other public sector settlements and to compensate for inflation. The vice-chancellors were in agreement with AUT that it was unfair for members of university staff to have their pay further depressed in relation to other employees in public institutions. But they insisted that an award over 4 per cent, the amount allowed for in the government-set cash limits, could not be met without outside help.

Dr Albert Sloman, chairman of the Committee of Vice-Chancellors, pressed the case for more money in a meeting with Sir Keith Joseph on 25 June. In a further letter on 23 July, after the settlement of the award, Dr Sloman pointed out that the rising salary costs would mean a further running down of the university system. In his written reply Sir Keith Joseph made it clear that additional money would not be forthcoming.

Given the universities' stringent cash limits, the negotiations between AUT and the vice-chancellors left little room for manoeuvre. In giving a 5 per cent award the government's attitude seems to be, if the unions want more money, they know it may mean fewer staff.

Jane Wynn

US drug regulation

Congress finds FDA wanting

Washington

Eli Lilly and Company has repeatedly failed to report adverse findings about drugs it was testing or marketing, according to Food and Drug Administration (FDA) documents made public last week. Sales of one of the drugs mentioned in the documents, Oralflex (benoxaprofen), were suspended last week in both Britain and the United States after a review of British data revealed 61 deaths associated with its use (see box).

The allegations against Lilly are spelled out in a series of internal FDA memoranda released by the House intergovernmental relations subcommittee, which was holding hearings on FDA's proposed streamlining of the new drug evaluation process.

One of the most serious charges is that "data for benoxaprofen appear to have been deliberately withheld, thus biasing the NDA (new drug application) in favour of approval". The investigator who reached that conclusion, Dr Michael Hensley, recommended last September that FDA "consider prosecution of appropriate Lilly employees".

Dr Hensley's investigation revealed in particular that 65 adverse effects that occurred during the clinical trials of Oralflex were not reported to FDA, even though they were apparently related to the drug, and were in Lilly's files when the

application was made in December 1979. These effects were principally photosensitivity and onycholysis (loosening of the fingernails), and all occurred after the "cut-off date" of November 1978 that Lilly had set on data that were to go into their application. The cut-off is apparently a usual procedure.

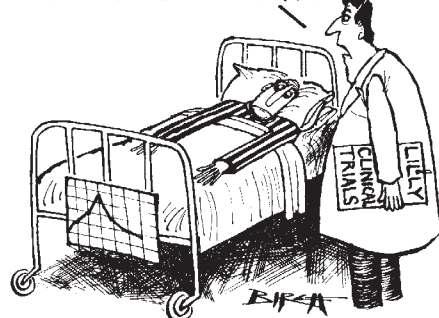
But the Lilly employee in charge of the application, Dr H.A. Bartlett, did review the adverse effect reports from trials that continued after the "cut-off date", and included "serious" reactions in the application, according to a memorandum written by Dr Hensley on 16 September 1981. This memorandum then states that Dr Bartlett instructed an employee "not to report others (such as onycholysis and photosensitivity) which he allegedly felt were, because of their frequent occurrence, no longer alarming" (*sic*). In fact, the new data showed a sharp increase in the incidence of these side effects. A letter from Dr Marion Finkel, then associate director for new drug evaluation, to R.D. Wood, chairman of the board of Lilly, on 12 March this year charges that "the consequence was a biased presentation clearly more favourable to the drug than was warranted by the data".

Lilly's answer is that FDA regulations simply do not require applications to include data on all adverse reactions

observed after the cut-off date. At the subcommittee hearings, Dr Robert Temple, acting director of the office of new drug evaluation, agreed. He called it a "defect in the regulations".

A similar mix-up occurred over the reporting of four deaths from jaundice associated with the drug. Attention was focused on this matter by reports (Goudie, E.M. *et al. Lancet* i, 959; 1982/Halsey, J.P. & Cardoe, N. *Br. med. J.* 284, 1365; 1982.) of several jaundice deaths in Britain associated with Oralflex. On 23 June, FDA officials met Lilly officials to discuss the matter, and "expressed surprise that (American) cases of jaundice had not been submitted prominently to the NDA (new drug application) prior to its approval".

"I'M AFRAID YOU HAVE
DOW JONES SYNDROME."



Lilly officials claimed that they had in fact submitted two reports to the IND file before approval. At the subcommittee hearings, FDA officials said they had had no way of checking that, since there was a six-month backlog in processing the IND files. They said they have since found that all four cases were reported to FDA by Lilly — but that FDA reviewers apparently approved the application without seeing the reports. FDA Commissioner Arthur Hayes Jr says that it "wouldn't have made any difference" in the approval, since the reviewers knew about the problem.

Dr Hensley's memorandum of 29 September 1981, in which he recommended possible prosecution of Lilly employees, referred to three other drugs about which he said "allegations . . . that Lilly has repeatedly failed to make required reports of important adverse findings" had been "confirmed" by FDA investigators. About aprindine, an anti-arrhythmia drug, Dr Hensley found that "Lilly employees may have engaged in highly objectionable, perhaps ethically questionable practices in their handling of the aprindine clinical programme". Furthermore, Lilly apparently ignored findings from its own experiments with dogs of a possibly lethal side effect of the drug, fibrillation. It was not until January 1974, after four patients in the aprindine clinical trials had died, that the dog studies were reviewed and finally reported to FDA on 4 February 1974. An FDA memorandum dated 4 February 1982 recommends that Lilly be issued a "Notification of Adverse Findings" for failing to incorporate the data from the

Arthritis drug proscribed in Britain

A voluntary decision by Eli Lilly to withdraw the anti-arthritis drug benoxaprofen from sale in all countries came soon after the imposition of a 90-day ban on UK sale by the Committee on Safety of Medicines (CMS). The Department of Health withdrew the product licence for the drug (sold under the trade name Opren in the United Kingdom) to allow time to assess the alarming total of 3,500 reports of serious side effects, especially in the elderly.

Benoxaprofen had rapidly become one of the most prescribed of anti-rheumatic drugs since the UK product licence was issued in 1980, and the CMS review cites 61 deaths associated with its use. In the United States the drug (sold as Oralflex) was given FDA approval only in April of this year, but already at least 11 deaths have been linked with benoxaprofen, again mostly in elderly patients and mostly involving liver or kidney damage.

In withdrawing the drug, Eli Lilly was at pains to explain that it still believed benoxaprofen to be safe and effective if used as directed. But after seven years of development before being allowed on the market, the question of why possible contraindications seem to be emerging only at this stage will be taxing regulatory authorities and Lilly's medical teams on

both sides of the Atlantic.

Benoxaprofen is undoubtedly effective in many cases of rheumatoid arthritis and osteoarthritis but it is probably not irreplaceable — other non-steroid anti-inflammatory agents with similar activity include aspirin, naproxen, ibuprofen and fenoprofen. For Eli Lilly, though, the whole episode is a major setback. Even if benoxaprofen is allowed back onto the market, the damage will have been done and doctors will be reluctant to prescribe it and patients to take it.

The latest events surrounding benoxaprofen provide another reminder of the high risks involved for companies attempting to reap the high rewards that accompany a successful new drug launch. Only two weeks ago the London Stock Market witnessed extraordinary fluctuations in the price of shares in the pharmaceuticals company Glaxo. High hopes of massive profits from sales of the new anti-ulcer drug Zantac (ranitidine) were dampened when a few reports of possible side effects were publicized with little analysis in the financial press. Share prices recovered when Glaxo and others pointed out the relatively minor nature of the reported effects, but predictably the price has not matched the high levels seen before the fracas.

Charles Wenz

animal studies into the clinical protocols and failing to report properly to FDA.

Dr Hensley's memorandum of 29 September 1981 concludes that for a third drug, drobuline, proper case report records were not maintained, and protocols were not followed. In the case of the fourth drug, monensin, the FDA documents charge that Lilly failed to report adverse effects in animals and humans exposed to the drug, or delayed reporting these effects

FDA regulations

Saving time, but carefully

Washington

The Food and Drug Administration (FDA)'s plans to relax some of its requirements for new drug applications came under congressional scrutiny last week during two days of hearings that also raised serious questions about Eli Lilly and Company's reporting of adverse effects of its drugs (see p.597).

FDA Commissioner Arthur Hull Hayes Jr, testified that the proposed changes in FDA procedures should shorten the approval process for the average application by six months. It now takes nearly two years. An FDA spokesman said, however, that the new procedures would probably have little effect on applications for drugs deemed important, since they are already given expedited treatment.

Representative L. H. Fountain (Democrat, North Carolina), chairman of the House subcommittee that conducted the hearings, focused strongly on two of FDA's proposed changes. One would drop the requirement that drug companies submit the detailed "case report forms" from clinical trials of the drug; the other would allow FDA to approve a new drug application on the basis of foreign studies.

At present, applicants are required to turn over to FDA all case report forms. These are the reports made by the clinical investigators on each patient; according to FDA, they make up 70 per cent of the applications now, often running into hundreds of volumes. FDA is proposing that, instead, the drug companies should be allowed to submit tabulations of the raw data, and only submit the case reports for cases that raise significant safety questions, such as patients who died, or dropped out of the study because of an adverse effect. The companies would still have to supply the case reports if requested by FDA.

Dr Robert Temple, acting director of the office of new drug evaluation, assured the subcommittee that FDA would not lose anything in the change. But detailed reports "will still be asked for as they're needed", he said; and Commissioner Hayes argued that tabulation of the raw data is "more consistent with current scientific practices".

Subcommittee staff members, however, noted that two in-house reviews at FDA found tabulations which did not agree with the case reports they were supposedly

by as much as 23 months. Lilly was issued a notice of adverse findings on monensin on 6 July 1981.

At the subcommittee hearings, Lilly issued a statement to reporters denying the charges. "Eli Lilly and Company takes vigorous exception to any implication that it withheld data, maintained inadequate records, or failed to comply with the requirements of the FDA".

Stephen Budiansky

drawn for. They were also concerned that FDA reviewers might be intimidated by the prospect of having to make a special request for the case reports, if for no other reason than the time it would take.

On the issue of foreign data, FDA officials similarly tried to be reassuring that the proposed changes would not undermine FDA's ability to make a thorough evaluation. FDA rules now allow foreign studies to be accepted if the investigators are "well-qualified" and if they make background data available to FDA. But in almost all cases, at least one domestic study is also required.

That would change under the new rules. A drug could be approved solely on the basis of foreign data; Hayes suggested that this would be especially important when requiring domestic trials would "cause an unjustifiable delay in the drug's availability to the public", would result in "unnecessary or duplicative testing", or would present an "unnecessary burden on the drug sponsor".

Foreign data would still have to meet US standards and be the product of "investigators of recognized competence". Critics worry that standards will nonetheless be lowered. Dr Sidney Wolfe of Ralph Nader's Health Research Group, said, "The main problem with the use of foreign data is that the drug laws and the protection of human subjects are weaker everywhere in the world" than they are in the United States. And according to Dr John Nestor, a retired FDA employee who worked for many years reviewing drug applications in the agency's cardio-renal drug division, the main effect of the change will be that "the drug companies will be getting their studies done in Mexico and Canada and everywhere else because it's easier to escape surveillance by FDA". At the subcommittee hearing, Representative Fountain released evidence that FDA had encountered just such problems when it attempted to investigate studies done in Mexico and Canada.

The changes FDA is planning appear to enjoy support in Congress. But there are some reservations. Representative Elliot Levitas (Democrat, Georgia) enthused about the benefits of deregulation, and then implied that the only weapon against the drug companies is vigilance.

Stephen Budiansky

Venture capital investment

Now Monsanto

Britain now has one of the best environments in Europe for innovation, a director of the US chemicals company Monsanto said last week. And Mr Richard A. Onians has put Monsanto's money where its mouth is, investing £4.75 million in a new £9.7 million venture capital fund launched last week in London (see *Nature* 5 August, p.505).

Onians describes Monsanto's investment as a window on European technology, but what the company will see through it is mostly British work. The fund is to be managed by Advent Management, which already controls another £10 million fund, Advent Technology, now 15 months old and with ten British investments already under its belt. Monsanto will have no control over the new fund, but Advent Management will use Monsanto for technology assessment.

Monsanto itself seems to have been tempted to Britain for its "window" because of government willingness to allow foreign investment (France would not let Monsanto invest there, in spite of a desperate need to rebuild the French chemical industry), low capital gains taxes and because of what Onians called British inventiveness. There are probably plenty of potential British entrepreneurs as well, he thinks, if only the money is made available.

Sir Kenneth Cork, the accountant and ex-Lord Mayor of London who is chairman of the new fund, believes Britain could make good use of £500 million of venture capital, ten times the total probably now on offer. "But the Trades Union Council plan of £1,000 million from government and £1,000 million from industry just wouldn't work", he said; venture money needs to be hard to get.

Advent Management has certainly found it harder to raise the money for Advent Eurofund than it was two years ago to raise it for Advent Technology, an essentially similar fund. The fashion among finance houses and insurance companies for investing in such funds seems to have been short-lived, says Advent director David Cooksey.

University investment in high technology venture funds seems, however, to be new — new certainly for Cambridge (£500,000) and Oxford (£100,000). St Andrews, Imperial College London, the Nuffield Foundation and Boston University (Massachusetts) have also invested, reaching a total academic interest of £1.5 million. Some 20 other British universities were interested, said Cooksey, but they had not got the cash.

From the universities' point of view, these investments are dealt with like any other but offer a chance of protecting assets against inflation. Cooksey, however, clearly sees them as a window on potential invention, and this is bound to be

the case even though no formal arrangements have been made. There is no need for formal committees, Mr W. Hyde, Secretary of the Chest (finance officer) at the University of Oxford, says the "old boy network" will suffice. **Robert Walgate**

Polish students abroad

A helping hand

Polish University students stranded in Britain by the declaration of martial law last December should be able to continue their education in Britain under a scheme launched by a group of academics at the London School of Economics (LSE). Following a personal appeal to university principals by Lord James of Rusholme, thirty universities and colleges have offered free places to Polish students unable to continue their studies in Poland.

A preliminary count of students last spring suggested that between forty and fifty places would be needed. Since 22 July, however, when General Jaruzelski made it clear that there would be no early end to martial law, some fifty more students have applied for assistance.

Most of the students had come to Britain on temporary leave of absence from their universities in order to learn English. Many of them, at the same time, had been appointed as roving delegates of the Independent Students' Association (NZS), which hoped to build up strong ties with students unions abroad. NZS was outlawed last January (unlike Solidarity, which technically is only "suspended"), a factor which has undoubtedly influenced the students' decision to remain abroad.

Incorporating the Polish students into the British university structure involves a number of difficulties, even with science students where the curriculum differences are least. Poland has no bachelor's degree; university courses normally last five years, ending with a master's degree.

A compromise has been worked out, whereby students who should be beginning fourth or fifth year work in Poland will enter the second year in British universities, while their juniors will begin again from the beginning. The students themselves seem fairly happy with this plan, since a few terms of what is essentially revision will offset the difficulties of studying in English.

The LSE team, which is acting as an unofficial placement board, feels confident that places will be found for all Polish students who genuinely wish to continue their studies in Britain. The main problem is now that of maintenance; the team estimates that, to take advantage of the places so far offered, it will have to raise some £200,000 for the students' maintenance over three years. A charitable trust — the Polish Students' Appeal Fund — has been launched, and possibilities such as local authority rent rebates are being explored. **Vera Rich**

Nature guide to bio-riches

As a service to readers working in biotechnology, to those who may have invested in this new technology or who may regard its commercial fortunes as an indicator of the success with which bright ideas can be turned into commercial reality, *Nature* will in future publish a monthly listing of the stock performance of 15 representative biotechnology companies in the United States.

This scoreboard of performance will appear in the second issue each month, and will record the price of each of the stocks as traded on the last Friday of the month immediately preceding, the traded price at the end of the preceding month, and the high and low prices during the calendar year.

Where the stocks concerned are traded on the New York Stock Exchange or the American Exchange, the prices listed are those traded. For stocks traded over-the-counter, the prices listed are the bid prices.

Nature will also publish once a month a weighted index of the listed stocks, taking an initial value of 100 as of 25 June 1982. In the calculation of this index, the contributions of the 15 listed stocks to the index are weighted according to the total value of the issued stock.

Between the Fridays of 25 June and 30 July, the *Nature* Biotechnology Index increased from 100 to 102.7.

The data (as of 25 June 1982) on which these calculations will be based are shown in the following table.

Market value of biotechnology stocks, 25 June 1982

	Outstanding shares (millions)	Closing price 25/6/82 (\$)	Total market value (\$ million)	% of all total market values
A.B. Fortia	19	23.375	444.1	21
Bio-Response	5.5	4.25	23.4	01
Cetus	22	8.0	176.0	08
Collaborative Research	8	8.0	64.0	03
Collagen	5.1	17.875	91.2	04
Damon	6.6	6.5	42.9	02
Enzo-Biochem	5.0	14.75	73.8	04
Flow General	8.0	8.5	68.0	03
Genentech	8.1	32.75	265.3	13
Hybritech	8.8	12.5	110.0	05
Molecular Genetics	4.0	8.5	34.0	02
Novo Industri A/S	16.6	38.25	635	30
Monoclonal Antibodies	2.0	9.5	19	01
Genetic Systems	13.7	2.75	37.7	02
Bio Logicals	5.9	2.75	16.2	01
Total			2,100.6	100

From time to time, *Nature* will also publish graphical analyses of the performance of stocks of particular interest, perhaps because the corporation is going through an eventful period or because the industry as a whole is at some turning point.

The stocks listed have been selected from among the much larger number for which

data are available on somewhat arbitrary criteria — because of the volume of transactions, for example. It is intended to review the listing from time to time.

Nature is grateful to E.F. Hutton and Co., Inc., for advice on the design of this service and for assistance with the compilation of the data. Suggestions from readers will be welcome.

Performance of biotechnology stocks, 30 July 1982

1982 high	1982 low		Close previous month	Close July 30	Change
28	16 ¹ / ₈	A. B. Fortia	23 ³ / ₈	25 ³ / ₄	+ 2 ³ / ₈
7	3 ⁵ / ₈	Bio-Response	4 ¹ / ₄	4 ⁷ / ₈	+ ⁵ / ₈
14 ¹ / ₈	8	Cetus	8	9	+ 1
11	6 ¹ / ₈	Collaborative Research	8	8 ³ / ₈	+ ³ / ₈
21 ⁷ / ₈	14 ³ / ₄	Collagen	17 ⁷ / ₈	17 ³ / ₄	- ¹ / ₈
8 ⁷ / ₈	5 ³ / ₄	Damon	6 ¹ / ₂	6 ¹ / ₄	- ¹ / ₄
17 ¹ / ₄	11 ¹ / ₄	Enzo-Biochem	14 ³ / ₄	11 ¹ / ₄ *	- 3 ¹ / ₂
28	6 ⁵ / ₈	Flow General	8 ¹ / ₂	7 ⁵ / ₈	- ⁷ / ₈
37 ³ / ₄	26	Genentech	32 ³ / ₄	30 ³ / ₄	- 2
17 ⁷ / ₈	9 ⁵ / ₈	Hybritech	12 ¹ / ₂	13 ³ / ₄	+ 1 ¹ / ₄
9	6 ¹ / ₄	Molecular Genetics	8 ¹ / ₂	6 ³ / ₈	- 2 ¹ / ₈
44 ⁵ / ₈	34 ⁷ / ₈	Novo Industri A/S	38 ¹ / ₄	39 ⁵ / ₈	+ 1 ⁵ / ₈
12 ³ / ₈	8	Monoclonal Antibodies	9 ¹ / ₂	9 ¹ / ₄	- ¹ / ₄
3 ³ / ₈	2 ¹ / ₄	Genetic Systems	2 ³ / ₄	2 ³ / ₄	0
8	2	Bio Logicals	2 ³ / ₄	3	+ ¹ / ₄

Close of month prices at the close of business on the last Friday of the month. Where stocks are traded over the counter, the price quoted is the bid price. For stocks listed on the American and New York Stock Exchanges the price quoted is the actual transaction price.

*High or low for the calendar year.

CORRESPONDENCE

Celltech and MRC

SIR — The House of Commons Select Committee's remarks (*Nature* 5 August, p.505) on Celltech's links with the Medical Research Council (MRC) raise some important issues:

(i) The cost of developing a discovery and bringing it to the market often makes a degree of monopoly essential if it is to be worthwhile for anyone to take on the development risk. On the other hand, giving one body monopoly rights for all inventions for all time may lead to stultification. So a good balance between exclusivity and competition needs to be struck.

(ii) Industry and academia quite rightly differ in their aims and ways of doing things, so collaboration between them will not be productive unless mutual understanding about aims and methods reaches a high enough level. This probably requires individuals and organizations to work together quite closely over a period of time.

The agreement between Celltech and MRC gives Celltech only a first option — MRC must always be satisfied with the commercial plans. It has other features novel in the United Kingdom which bring MRC and Celltech scientists closer together. Experimentation with a variety of frameworks for industrial/academic collaboration is highly desirable and that between MRC and Celltech may turn out to be worth copying. It has been operating for less than two years but has already led to commercial success, for example, in anti-interferon. The time is perhaps too short to have tested the ideas fully but if a review at this stage could be helpful to others, I would be happy to see it.

G.H. FAIRTLOUGH

Chief Executive,
Celltech Limited,
Slough, Bucks, UK

The numbers game

SIR — Dr H. H. Rossi (*Nature* 22 July, p.320) makes a plea for a modification of our way of writing quantities in the SI system.

This raises various issues. The numbers we write in English speaking countries as 1.234 and 1,234 tend to be written the other way round in continental Europe (with a "decimal comma" instead of a decimal point, and a dot for thousands). This is a dangerous confusion, and one has to be thankful that so far it hasn't resulted in catastrophe. What makes it worse is that especially in handwriting (and sometimes also in print) not only may dots and commas be difficult to distinguish reliably, but also they may be so faint as to escape notice altogether.

What is wanted is a good clear symbol for the decimal point, which ideally should be already present on all ordinary typewriters, and not already used as a symbol for a unit of measurement or easily confused in handwriting with such a symbol.

About the only symbols satisfying all these conditions are "&" and the letter "u". If we wrote "2&34" or "2u34" for "2.34" there would be little danger of confusion. There is also a lot to be said for the admirable brevity of the occasional continental habit of writing, say, 2.34 m as "2m34". It is quicker to write and to say, and is unambiguous. Brevity is a

great help to clarity. Although "0.700" means exactly the same as ".7", it is ".7" which makes the quickest and strongest impression on the mind.

For representing numbers in so-called scientific notation, I would suggest that we write, for example, 2(6)34 for 2.34×10^6 . This notation is brief and more flexible than the traditional one. There is no objection to writing 1(3)234(0)567(-3)g if one wishes, showing simultaneously that this is 1.234567 kg, or 1234.567 g, or 1234567 mg, according to which units are most convenient. In addition, 2(3), meaning 2000, could be written unambiguously as 2(3 with the second bracket understood, again encouraging brevity, and similarly 6(14) (= .14 million) could be written 6)14.

Of course some problems remain; for example most computers already use brackets in at least two other senses already. Perhaps a convention should be made of using square brackets in computer programs, writing 2[68]34 for what is now more clumsily written 2.34E06 or 2.34×10^6 . But these problems should resolve themselves in due course.

CEDRIC A.B. SMITH

Department of Genetics and Biometry,
University College London, UK

Drugs as carcinogens

SIR — The main impression given by Dr M. Weatherall (*Nature* 1 April, p.387) is that the maintenance and improvement of the health of humans should be based principally on the development of new drugs, and that any delay, such as compliance with government regulations, in the introduction of a new drug would result in an increased loss of human health.

Fortunately the approach of regulatory agencies differs from that of the pharmaceutical industries. The agencies attempt to reduce the occurrence in the population of side effects of drugs and to assess the benefits of drug therapy. The causes of the major diseases are only partly known, and an incomplete rationale behind the therapy for such diseases results in what L. Thomas has called "halfway technology", involving very high costs and relatively unsatisfactory results. An additional problem is that the effectiveness of medicines is oversold².

Improvement of human health in the future will depend on greater knowledge of the aetiopathogenesis of diseases, on improvements in social conditions and on more specific treatments¹⁻⁷. Priority should be given to maintaining a proper balance among these different approaches.

There are certainly limitations in the capacity of *in vitro* assays and *in vivo* carcinogenicity tests for the detection of possible carcinogenicity in humans. However, the intelligent use of such tools has proven valid in predicting carcinogenic effects in man⁸. Of the drugs mentioned by Dr Weatherall, phenacetin induces tumours of the urinary system of rats, and epidemiological studies⁹ show the same organ system to be the target for its carcinogenic effect in humans; there is also evidence that phenacetin is a mutagen in bacteria and mammalian cells⁹. Azathioprine, chlorambucil and

cyclophosphamide, known to be responsible for an increased risk of developing tumours among patients undergoing kidney transplantation, are carcinogenic in experimental animals and mutagenic in various systems⁹. Metronidazole is carcinogenic in mice and rats and mutagenic in bacteria and fungi, while the available epidemiological data are inadequate for a proper assessment of its carcinogenicity in humans. The following drugs or treatments, proven to be carcinogenic in humans, were also found to be carcinogenic in experimental animals: chlornaphazine, myleran, melphalan, methoxsalen (in combination with ultraviolet A irradiation, PUVA), and diethylstilboestrol. Chlornaphazine, myleran, melphalan and PUVA are also mutagens⁹.

Thus evidence of an adverse effect observed in experimental systems should not be underestimated, and in some cases, it can have value as a predictor for a similar effect in humans. This value should increase as more is known of the mechanisms of carcinogenesis.

A summary by Dollery¹⁰ of a recent meeting concludes that there is still a tendency to underestimate risks and to overestimate benefits when evaluating drugs. It seems reasonable that the experimental tests to detect possible side effects of drugs should remain a part of the process of the development of a drug, one of the main aims being the provision of reliable information on which a risk-benefit evaluation can be made. The argument that this process is expensive is tenuous, when one considers that approximately 10 per cent of the budget of the pharmaceutical industry is devoted to research and development and 20 per cent to the marketing of drugs. It is worth mentioning that recently, WHO has listed a total of approximately 200 essential drugs to help in the maintenance of health¹¹, a very small number compared with the number of drugs now on the market.

R. MONTESANO

International Agency for Research on Cancer,
Lyon, France

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Correction

THE letter from J. A. Nicoll in *Nature* of 10 June (p.450) on "Logarithmic SI" contained the following errors. In line 10 p2.3 should read p2.3; in line 25 pDO stands for density, 1 kg m⁻³ not m⁻²; in line 29 pL*8.5 should read pL*8.5; and in line 30 pL*9 should read pL*9.

NEWS AND VIEWS

What are quarks made of?

from Louis Lyons

In 1964, the properties of the hundreds of 'elementary' particles then known were explained in terms of just three quarks. But, since then, the number of quark types has grown. Added to the original up, down and strange quarks are the charmed and bottom quarks and a sixth, the top quark, is presumed to exist. Furthermore, each quark is now known to exist in three different 'colours' and a further eight particles, the gluons, are required to mediate the binding forces between them.

History thus seems to be repeating itself for in each past attempt to explain the ultimate structure of matter, an initial reduction in the number of entities required — be they atoms, nuclei or elementary particles — has been followed by further discoveries of similar objects until, finally, the cycle has repeated itself and another level of substructure has been proposed at which the number of basic particles has again become small. Are we to believe then that quarks are not, after all, elementary?

In addition to quarks, which are the constituents of the strongly interacting particles mentioned above, the world also contains leptons. The best known are the electron and its neutrino, but nature has twice replicated them with the muon and the tau leptons, which both appear to be simply heavier versions of the electron and both of which have their own neutrino. Some of the properties of leptons and quarks are listed in the table.

The repetitive similarities of both the quarks and leptons suggest that they be grouped in families or generations with increasing mass, in a manner somewhat reminiscent of the chemical periodic table. This in itself is suggestive of another level of substructure for quarks and leptons. Again, further generations may also exist, still waiting to be discovered.

A wide variety of suggestions concerning substructure has been made in the past couple of years¹. Any prospective model must address a number of issues: (1) How many preons (the name given to the substructure elements) make a quark or

lepton? (2) Should quarks and leptons be constructed entirely from the same preons or should at least one preon in a quark and in a lepton differ? (3) Are the preons bound together by gravity, electromagnetism or some new extra strong force? (4) What is the energy scale Λ or distance scale r on which quarks and leptons are composite? Popular values congregate around about 10^3 GeV or 10^{15} – 10^{19} GeV (equivalent to $r \sim 10^{-17}$ cm or 10^{-39} – 10^{-33} cm [the mass of the proton is ~ 1 GeV]). (5) What makes the different generations, and how many are there? Are the higher ones simply some form of excitation of the first, do they contain extra combinations of preons, does one preon exist in a few different forms, do they correspond to a fine structure type splitting, or something entirely different? (6) What is a plausible scenario for making the composite systems so light on the scale of Λ , and yet still have an interesting mass spectrum (see Table 1)? (7) What other dynamical predictions can be made?

One model² has preons T and V of charge $1/3$ and zero respectively. The first generation quarks are TTV and $\bar{T}\bar{V}\bar{V}$ composites while the electron and its neutrino are $\bar{T}\bar{T}\bar{T}$ and VVV (the bar above a preon denotes its antiparticle). In another model³ the preons are labelled h_i , w_i and c_k , where h_i , h_j and h_k determine the generation, w_1 and w_2 are two different charged preons, and c_1 , c_2 and c_3 give the three colours to the quarks while c_0 is the charged colourless lepton's preon. Here the blue up and down quarks are $h_1 w_1 c_3$ and $h_1 w_2 c_3$, while the μ^- is $h_2 w_2 c_0$.

Most models nowadays try to do more

than simply obtain the quantum numbers (that is spin, charge, colour, generation number, etc.) of the observed quarks and leptons. However, there are not many testable predictions and those that exist are often not very specific to substructure models. Thus many composite models suggest that neutrinos should not be exactly massless and that the proton should decay at a very slow rate; but other non-composite models make these predictions as well. A significant advance would be if a mass formula were deduced, which enables as yet undiscovered quarks and/or leptons to be predicted.

The obvious question is whether there is any experimental evidence in favour of quarks or leptons being composite. The answer to date is 'No'. The relevant experiments can then be used to set lower limits on Λ .

For example, the magnetic moments of truly elementary particles can be predicted from quantum electrodynamics. Substructure effects are likely to produce deviations; the composite nature of atoms, nuclei and even 'elementary' particles results in their magnetic moments differing from the predictions for point-like objects. The fact that the magnetic moment of the electron and muon agree with point-like values, to accuracies of 1 in 10^9 and 1 in 10^8 respectively, results in stringent constraints on their composite structure.

Other sources of information on Λ include reactions such as $e^+e^- \rightarrow \mu^+\mu^-$, e^+e^- annihilations producing strongly interacting particles, and the scattering of muons or neutrinos off nuclei. In all cases

Table 1 Properties of quarks and leptons

	Up(u)	Down(d)	Electron(e ⁻)	Neutrino(ν_e)
Generation 1				
Mass (GeV c ⁻²)	0.3	0.3	5×10^{-4}	$<5 \times 10^{-8}$
Generation 2				
Charm(c)		Strange(s)	Muon(μ^-)	Neutrino(ν_μ)
Mass (GeV c ⁻²)	1.5	0.5	0.1	$<6 \times 10^{-4}$
Generation 3				
[Top(t)]		Bottom(b)	Tau(τ^-)	Neutrino(ν_τ)
Mass (GeV c ⁻²)	?	5	1.8	<0.3
Quark or lepton	Quark	Quark	Lepton	Lepton
Electric charge	$+2/3$	$-1/3$	-1	0
Colour	Red, green, blue		Colourless	Colourless
Directly observed?	No		Yes	Yes
Forces	Strong		Electromagnetic	Weak
	Electromagnetic		Weak	
	Weak			

Louis Lyons is in the Department of Nuclear Physics, University of Oxford, Keble Road, Oxford OX1 3RH.

to date, the predictions based on a point-like nature of quarks and leptons agree with experiment.

The above data can be used to deduce that Λ is greater than 600 GeV. Thus quarks and leptons, if they are composite, must be very tightly bound systems, since their masses are so much smaller than Λ .

There are other reactions and decays which are expected in some models of substructure. Thus if the muon is an excited state of an electron, the decay $\mu \rightarrow e\gamma$ should occur. Similarly, if the quarks in a proton share a common substructure with leptons, proton decay becomes possible. Again none of these types of processes has been seen, but because of the model dependence of the predictions, no firm limit on Λ can be deduced.

Theoretical speculation to date has thus been unhampered by any positive

experimental evidence. Models have thus become exercises in self-consistency with the main constraints coming from the need to produce almost massless composite states and the necessity of not violating any experimental bounds which have been set in attempts to observe substructure effects.

The main hope for the future is that experiments at the new accelerators becoming available during this decade may reveal direct evidence for quarks and/or leptons possessing substructure. This would then change the motivation for the subject from the realms of aesthetics, history, psychology and sociology to that of physics. $\square$

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Proteins containing nickel

from Andrew J. Thomson

NICKEL long stood out among the six metallic elements of the latter half of the first transition series, namely, Mn, Fe, Co, Ni, Cu and Zn, as being apparently without biological function apart from its toxicity. The situation has changed rapidly in the last few years¹. Nickel is now known to be a nutritional requirement for many eukaryotic and prokaryotic organisms.

The element has been shown to be a component of urease in plant cells growing on urea as sole nitrogen source², to be present in the hydrogenases from several types of bacteria including those which can perform the Knallgas reaction ($2\text{H}_2 + \text{O}_2 \rightarrow 2\text{H}_2\text{O}$)³ and the sulphate-reducing bacterium *Desulphovibrio gigas*^{4,5}, to be in the enzyme carbon monoxide dehydrogenase in acetogenic bacteria⁶ and, lastly so far, in a low molecular weight tetrapyrrole present in methanogenic bacteria^{7,8}. Other roles for nickel are also apparent in, for example, a plant such as *Alyssum* which accumulates large amounts of nickel⁹ and in a strain of *Bacillus cereus* which forms a nickel-containing intracellular pigment that is probably a protein¹. It has also been suggested that nickel is involved in endospore formation of these bacteria¹.

Urease catalyses the hydrolysis of urea in the reaction



The enzyme from jack bean contains about 6–8 mol of nickel per mol of protein (molecular weight 480,000). The metal appears to be in the oxidation state +II (ref. 2). This contrasts with the nickel centres recently discovered in some hydrogenases from *Methanobacterium thermoautotrophicum*³ and from *D. gigas*

which have been ascribed to nickel in the trivalent or +III state since electron paramagnetic resonance (EPR) signals are readily detectable^{4,5}. These discoveries stemmed from earlier observations that nickel is required for the biosynthesis of hydrogenase in 'Knallgas' bacteria¹⁰ and that there is an EPR signal in both soluble and membrane-bound fractions from the methanogen *M. bryantii*¹¹. The assignment of the EPR signals in this latter case to Ni III, by Lancaster¹¹, was a bold one. The Ni III/Ni II potential of inorganic complexes of peptides was known to be above 0.7V, making Ni III an unlikely species in a hydrogenase¹².

However, Lancaster was persuaded of this assignment for the following reasons¹¹. The signals occurred at $g = 2.30, 2.23$ and 2.02 , all above the free-electron value and therefore unlikely to arise from haem. They were observable up to 80K and were quite unlike those of any known iron-sulphur centres. The g -factors pointed to a metal ion with the electronic structure low-spin d^7 . The lack of nuclear hyperfine structure eliminated cobalt(II). This left Ni III as the only candidate, however unlikely on chemical grounds.

It has since been shown that the hydrogenase from *M. thermoautotrophicum*³ also gives the characteristic nickel EPR signals at g -factors of 2.3, 2.2 and 2.0. Studies of the hydrogenase purified from cells grown on a medium enriched in ^{61}Ni , an isotope with a nuclear spin of $3/2$, showed nuclear hyperfine structure on the lines at 2.23 and

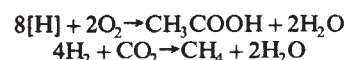
2.01, which proved beyond doubt that these signals arise from nickel. A spin quantification of the nickel EPR signals gives a value in close correspondence with the chemically determined nickel content, being about 0.6 mol Ni per mol enzyme.

The assignment of the nickel ion to the trivalent state has now been confirmed by a study of the EPR spectrum of the periplasmic hydrogenase from *D. gigas*^{4,5}. A redox titration of the $g = 2.24$ signal from nickel in this enzyme shows that it is lost on reduction with a mid-point potential (E_m) of -145 mV at pH 7.2 (ref. 5). Moreover the E_m value is pH dependent, showing that one proton is picked up during reduction⁵. These potentials are higher than those of the $2\text{H}^+/\text{H}_2$ couple by about 280 mV, raising obvious mechanistic difficulties for the involvement of the nickel centre directly.

The low redox potential for the Ni III/Ni II couple also raises interesting questions about the likely coordination site of the metal ion in the protein. Several nickel-peptide complexes have been prepared which involve a rather narrow range of amino acids, including leucine, phenylalanine, histidine and aspartic acid¹². The complexes can be electrochemically oxidized to the trivalent state at E_m values of between $+0.79$ and $+0.96$ V. The oxidized state appears to involve the deprotonated peptide linkage. The EPR properties of the Ni III peptides are not dissimilar from those of the protein-bound Ni III ion. However, the remarkably different redox properties argue for a very different site of ligation in the protein possibly involving sulphur ligands.

The periplasmic hydrogenase from *D. gigas* also contains about 12 Fe atoms per mol, undoubtedly arranged into several separate iron-sulphur centres⁴. EPR signals are also observed from these centres both in the oxidized and reduced states of the enzyme. Curiously, however, Albracht *et al.* could detect no iron-sulphur EPR signals in the hydrogenase purified from *M. thermoautotrophicum*³. All hydrogenases purified before this report contain iron-sulphur centres. The authors do not yet discount the possibility that the iron-sulphur centres could have been lost during aerobic purification of the enzyme. If not, this will be an intriguing case of a hydrogenase containing only nickel as its metal component.

Pre-dating much of this work on the EPR detection of Ni III in hydrogenases were the observations that nickel is necessary for the biosynthesis of carbon monoxide dehydrogenase in acetogenic bacteria such as *Clostridium thermoaceticum*, *C. acetium* and *Acetobacter Woodii*¹³ and also for the growth of methanogenic bacteria with H_2 plus CO_2 as sole energy source¹⁰. The two classes of reaction being catalysed are respectively



Andrew J. Thomson is in the School of Chemical Sciences, University of East Anglia, Norwich NR4 7TJ.

From the methanogenic bacterium *M. thermoautotrophicum* it is possible to isolate a yellow, low molecular weight nickel-containing compound with an absorption maximum at 430 nm, hence named factor F_{430} (ref. 7). This factor has been found in every methanogenic bacterium so far examined⁸. The extinction coefficient at 430 nm is high, $\sim 23 \times 10^3$ per cm per mol Ni. The exact structure of this pigment has not yet been elucidated but there is evidence from the biosynthetic incorporation of δ -amino laevulinic acid that F_{430} is a nickel tetrapyrrole¹⁴. Chemical degradation products of F_{430} have appreciable absorption in the region 500–600 nm and react with cyanide. It has been suggested that the spectrum resembles that of vitamin B_{12} with, presumably, the implication that the nickel species is related to nitrin, the nickel analogue of corrin¹. There is evidence of a mechanistic kind that the carbon monoxide oxidation in acetogenic bacteria may also involve a prosthetic group similar in structure in vitamin B_{12} but with nickel in place of cobalt¹⁵. There is a very recent report that the co-enzyme of the purified methyl reductase of *M. thermoautotrophicum* is F_{430} , with a stoichiometry of 1 mol of nickel per mol of F_{430} and 2 mol of F_{430} per mol of

methyl reductase¹⁶.

Thus it is now clear that there are at least two new classes of nickel-containing enzymes: the nickel-containing hydrogenases and those with a nickel tetrapyrrole-related co-factor. Much work on their structural elucidation needs to be carried through. □

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telescope mirror than the traditional test of 'whether the dome rail is wet' needs to be developed. A more professional approach would even benefit conventional observing with the observer present.

The financial benefits of remote observing are obvious, given rising air fares and falling communications costs. Advantages in flexibility might also be provided — the difficulties of scheduling simultaneous observations on different telescope and/or satellites might, for example, be eased. When some particularly demanding meteorological requirement is expected to be met for only a short time (for example, conditions dry enough to use an IR telescope in the 350 μ m 'window') it would be easier to put a different observer on the telescope if he did not have to travel so far to exploit this short period. Efficiency would also be improved as astronomers would spend less time travelling and recovering from jet-lag.

There are individual problems in communicating from different sites as reports by Paul Bryant (Rutherford and Appleton Laboratories) and Peter Kirstein (University College London) showed a somewhat anarchic situation in the communications field. There are differences in computer-to-computer communication protocols, in line tariffs and in regulations from country to country so that the best solution at one observatory might not apply at another. Obviously, however, common requirements exist and common solutions and standards are highly desirable as participants, looking ahead, foresaw a network for remote operation of telescopes linking Australia, Hawaii, Chile, continental United States, the Canary Islands and Europe. One solution to the present problem was to separate the difficulties of communication and control, defining interfaces between these segments only. Thus cheap entry to the field could be obtained with use of dial-up telephone lines, as at present, and when wide band lines or public packet switching networks become cheaper it would be possible to change to them with minimum disruption. The eventual use of wide-band 50 kbit lines was foreseen since that would allow near-real-time transmission of astronomical data from the telescope to the site of remote operations. In the meantime it seems most practical to progress using two to three dedicated telephone lines.

At the end of the meeting Alec Boksenberg, Director of the RGO, undertook to have remote operation from Herstmonceux of the 2.5 m Isaac Newton and 1.0 m telescopes on La Palma working by the end of next year. Obviously at this pace, the sky is the limit in the remote operations field. □

Remote operation of telescopes

from Michael Penston

BRITISH astronomers have recently moved to the fore in several branches of optical astronomy, thanks largely to the building of new telescopes in favourable sites abroad — in particular the Anglo-Australian Telescope at Coonabarabran, New South Wales, and the UK Infrared Telescope (UKIRT) on Mauna Kea in Hawaii. Access to the telescopes has, not surprisingly, so far depended on the opportunities provided by air travel. Now, astronomers have begun discussing whether modern communications technology offers an alternative way of working. Early in June experts in communications and computer technology came together with astronomers and builders of astronomical instruments at the Royal Greenwich Observatory (RGO) to discuss the feasibility of remote operation of telescopes*.

Remote operations of telescopes is possible and, indeed, has already been carried out in an experimental way by Kitt Peak National Observatory and UKIRT. The Kitt Peak 4 m and 2.1 m telescopes

have been used by observers at the end of telephone lines both from nearby downtown Tucson and at distant sites in Michigan and the East Coast of the United States. Three lines were used — one for voice, one for TV pictures needed by the astronomer to identify his target star and one to run a remote terminal linked to the computer in the telescope dome. UKIRT is running a similar system which offers the major advantage of being able to diagnose and solve instrumental problems from sea level without suffering the problems of lack of oxygen at the Mauna Kea telescope site 14,000 feet higher up.

There is little difficulty in sending the acquisition images of star fields. Images with 10,000–20,000 pixels with 3–4 grey levels transmitted each 10–20 seconds are perfectly adequate as this update rate is used by current integrating TV systems on telescopes. These could pass down a standard 9.6 kbit line even without data compression. In fact, the Kitt Peak experiment had found a 30 second update rate to be satisfactory. On the other hand, present astronomical practises regarding weather monitoring and protection of the instruments from damage were not so easily compatible with remote operation. A better way of determining whether the humidity is high enough to hazard the

*The two-day workshop on 'Remote operation of telescopes' was held at Herstmonceux Castle, home of the Royal Greenwich Observatory. Participants included representatives from UKIRT, Kitt Peak National Observatory, the European Southern Observatory and the European Space Agency as well as British astronomers and experts in computer networking.

Michael Penston is at the Royal Greenwich Observatory, Herstmonceux Castle, Hailsham, East Sussex BN27 1RP.

The biogeography of the Pacific Basin

from Jared M. Diamond

THE Pacific Basin includes the world's largest and deepest ocean as well as most of the world's islands. It constitutes the eastern half of the richest province of marine biogeography, and is bordered by five of the six provinces of terrestrial biogeography. Its biota includes textbook examples of adaptive radiation, such as the Galapagos finches, the honey-creepers and *Drosophila* of Hawaii, and the marsupials of Australia and New Guinea. Little wonder then, that it has long held a fascination for biogeographers.

The last major symposium on Pacific Basin biogeography was convened 21 years ago in Honolulu at the 10th Pacific Science Congress, by J.L. Gressitt¹. For the recent 10th annual meeting of the Association of Systematics Collections* Gressitt again organized a symposium on Pacific Basin biogeography at Honolulu's Bishop Museum. Sadly, the symposium took place without him — a month before the conference he died in an aeroplane crash in China.

The 1961 symposium was marked by polemical discussions about the relative contributions of long-distance overwater dispersal, former land bridges and continental drift to the founding of Pacific biotas. Proponents of one factor often dismissed the possibility of any significant role of the other factors in scathing terms. In retrospect, we can now see that the polemics arose because convincing evidence to settle the dispute was unavailable in 1961. At that time it equally strained one's credulity to accept that continental drift had occurred, or to accept that long-distance dispersal could explain everything in the absence of drift. In the following 21 years new types of evidence have made continental drift an accepted fact. Participants at the 1982 symposium took it for granted that drift and dispersal were both significant, and they proceeded without polemics to seek explanations for distributional patterns in terms of both.

Carson's studies on Hawaiian *Drosophila* provide a good example. In

Hawaii, this genus is represented by hundreds of endemic species, whose inter-relationships can be determined from the banding patterns of giant chromosomes². For all of the 26 large picture-winged species of Big Island, the nearest relative among the species on the older islands of Maui, Oahu and Kauai (respectively 1.8, 3.3 and 5.6 Myr old) has been identified. As Big Island is only 700,000 years old, these 26 populations (25 of them endemic to Big Island at the species level) must have arisen

Hawaiian high islands are merely the youngest in a chain of volcanoes formed by relative motion of the Pacific Plate and a volcanic hot spot, and subject to gradual erosion and submergence after formation. The Hawaiian low islands (11–27 Myr old) and the submerged Emperor Seamounts (37–70 Myr old) are the modern remains of older high islands on which the ancestors of the Hawaiian *Drosophila* and honey-creepers presumably made their initial colonization and then diverged.

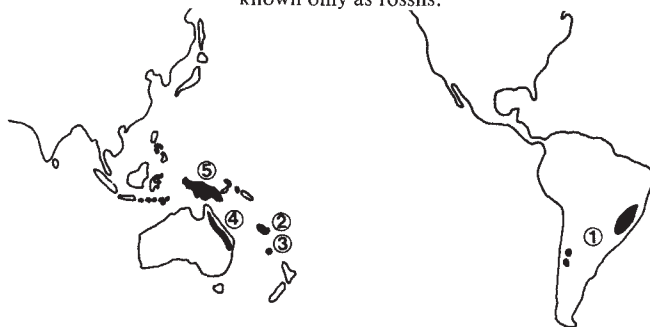
In contrast to this explosive radiation of *Drosophila*, some other taxa, such as ferns, bats, inshore marine fishes and molluscs, and plants of coral atolls, exhibit little or no local adaptive radiation and little local endemism beyond the species level on Pacific islands east of New Guinea. At the opposite extreme, achatinellid land snails have evolved numerous endemic genera on Hawaii, Juan Fernandez and the Australs, including nine endemic genera and one nearly endemic subfamily on the small (16 mi²) island of Rapa alone⁵! Numerous groups of insects and flowering plants are also prone to local endemism and adaptive radiation, while Pacific land birds have radiated only on Hawaii, the Galapagos and New Zealand and belong elsewhere mainly to New Guinea genera or closely related genera. Why such differences among taxa? A large part of the explanation is intertaxon differences in dispersal ability. Differentiation and radiation proceed rapidly when a rare colonist of a sedentary group, such as land snails, reaches an island. However, differentiation and radiation are inhibited by the rain of colonists in mobile species such

as ferns, with their wind-borne spores, or marine fish and molluscs, with their planktonic larvae. Another relevant factor is inter-taxon differences in propensity for evolutionary losses of dispersal ability^{6,7}: colonizing birds and insects often evolve to become literally flightless, and plants figuratively so, but there is no case of a flightless bat (how could one forage?).

A major contribution of continental drift has been to clarify how certain taxa of poor overwater crossing ability came to



Above, Pacific distribution of herons of genus *Egretta*, resulting from overwater dispersal that continues today. These birds are strong fliers and reach almost every scrap of land in the Pacific, no matter how remote. Herons have landed on ships in mid-ocean thousands of miles from land. Below, Pacific distribution of araucarias, resulting from continental drift. The huge cones and large seeds of these trees are unsuitable for overwater dispersal. Today, native araucarias are confined to South America (1), New Caledonia (2), Norfolk Island (3), Queensland (4) and New Guinea (5). After the breakup of the supercontinent Gondwanaland 50–100 Myr ago, araucarias survived on some of its fragments and were rafted by continental drift to their present locations. On other fragments, such as Antarctica, New Zealand, India and South Africa, araucarias became extinct and are known only as fossils.



within that time by overwater colonization from known sources, followed by reorganization of the gene pool (see J.S. Jones *News & Views* 289 743; 1981). One infers similarly rapid speciation in the Hawaiian honey-creepers (Drepanididae) and other Hawaiian taxa, and in the tree sunflowers of the Juan Fernandez islands (about 1–4 Myr old).

Yet biochemical dating indicates that the Hawaiian *Drosophila*³ and honey-creepers⁴ diverged from their mainland relatives at a date estimated at 15–42 Myr ago, considerably before the oldest of the existing Hawaiian high islands rose above the sea! The explanation is that the existing

*The participants in the symposium were P. Raven (Missouri Botanical Garden, St Louis), F. Kay and H. Carson (University of Hawaii, Honolulu), J. Diamond (UCLA), J. Stuessy (Ohio State University), E. Bousfield (National Museum of Natural Sciences, Ottawa), J. Holloway (Commonwealth Institute of Entomology, London), P. Morat (CRSTCM, Noumea) and F. Fosberg (US National Museum of Natural History, Washington DC).

Jared M. Diamond is Professor of Physiology at the University of California Medical Center, Los Angeles, California 90024.

have far-flung representatives on New Caledonia, New Zealand, Australia, New Guinea and South America. The numerous examples include southern beech (*Nothofagus*), hoop pines (*Araucaria*), marsupials, leptodactylid frogs, migadoline carabid beetles and peloridiid bugs. It was tacitly assumed without argument at the 1982 symposium, and hotly debated at the 1961 symposium, that these land masses were formerly joined in Gondwanaland and that these taxa were rafted to their present locations by the motion of Gondwanaland's fragments. Yet a mystery remains why the rafted survivors constitute such a selective fraction of Gondwanaland's former biota. With the doubtful exception of a flightless bird (the Kagu), New Caledonia has no Gondwanan relict vertebrates: in fact its only other flightless vertebrates are some lizards and one other flightless bird, endemic but recently derived. The sole candidates for Gondwanan relict vertebrates on New Zealand are its flightless birds, its two frogs and a primitive reptile (the Tuatara). How

did New Caledonia and New Zealand come to lack marsupials and other Gondwanan vertebrates that survived in Australia and South America? Could New Caledonia and New Zealand somehow have acquired Gondwanan trees without the vertebrates living in them, and Gondwanan insects without the vertebrates eating them⁸? Or, did most of the old vertebrates become extinct in the confined areas of New Caledonia and New Zealand, because vertebrates require larger areas for long-term survival than do many insects and plants? Could some of the flying birds of Australia be Gondwanan relicts, like the marsupials^{9,10}? □

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Warts and all

from M. Saveria Campo

THE last few years have seen a remarkable progress in our knowledge of papillomaviruses, as was very evident at a recent workshop* involving clinicians, pathologists and virologists and held in Freiburg. Papillomaviruses are the aetiological agents of papillomas or warts in their natural hosts, and are widespread and heterogeneous: so far twelve different types of human papillomavirus (HPV1-12) and six types of bovine papillomavirus (BPV1-6) have been characterized (Orth, Institut Pasteur; Jarrett, University of Glasgow).

Although originally benign, some papillomas can become malignant under the influence of environmental and genetic factors, as in the case of epidermodysplasia verruciformis lesions in humans¹, papillomas of the upper alimentary tract in cattle² and papillomas of the cottontail rabbit³.

In addition to these well documented cases, ample evidence was presented at the workshop of the possible pathogenic role of papillomaviruses in some anogenital malignancies in humans. Thus, HPV6 DNA has been found in several types of genital warts (Gissmann, University of Freiburg; Mounts, Johns Hopkins Medical School; Grussendorf, University of Aachen; De Villiers, Onderstepoort, Pretoria) and HPV particles and antigens have been detected in anal warts (Oriel,

University College London) and in several lesions of the uterine cervix (Pixley, University of Western Australia; Rilke, Istituto dei Tumori, Milan; Meisel; Morin, University of Quebec; Braun, Johns Hopkins Medical School). Both anal and genital warts have occasionally been found associated with malignant tumours.

In a particularly extensive survey of 250,000 unselected individuals, Meisel found that about two-thirds of the cases of mild and moderate atypia of the cervix are in fact papillomavirus-induced flat warts. In a more selected sample of cone biopsies, flat warts were frequently found associated with cervical dysplasia, *in situ* carcinoma and microinvasive carcinoma. These findings confirmed the view held by the clinicians and the pathologists present at the workshop that, although it is commonly accepted that severe atypia of the cervix is the result of direct progression from mild and moderate atypia, most cases of the latter are in fact papillomavirus lesions, and thus different entities from severe atypia. However, the question of whether or not there is a direct transformation from flat warts to frankly malignant structures is still open.

Perhaps the most striking and best known example of the malignant conversion of virus-induced tumours is provided by epidermodysplasia verruciformis (Orth; Jablonska, Warsaw Medical School). Several types of HPVs with different degrees of oncogenicity have been found in EV lesions, but despite this multi-

plicity, only HPV5 DNA is found in the squamous carcinomas, as multiple non-integrated episomes (Orth; Jablonska; Pfister, University of Freiburg; Faras, University of Minnesota).

Malignant transformation of papillomas is observed also in animals, where the synergism between the virus and other co-carcinogens can be studied experimentally.

Injection of bovine papillomavirus in the urinary bladder of cattle causes lesions which usually regress with time, but can occasionally progress to malignancy, particularly in animals eating bracken fern (Olson, University of Wisconsin), which contains both immunosuppressant and co-carcinogenic substances⁴.

Papillomatosis of the upper alimentary canal, adenocarcinomas of the large intestine, and transitional carcinomas and haemangiosarcomas of the urinary bladder have been experimentally reproduced in cattle injected with virus and either fed with bracken or immunosuppressed with azathioprene. Non-integrated multiple copies of viral DNA have been found in both the experimentally induced bladder cancers and in naturally occurring ones (Jarrett).

Multiple copies of non-integrated non-methylated BPV DNA have been found also in *in vitro* transformed cells (Moar, University of Edinburgh; Pfister). Non-integration and non-methylation of papillomavirus genomes in tumours and transformed cells seems to be the general rule. One exception, however, is provided by papillomas and carcinomas induced by the cottontail rabbit papillomavirus in the domestic rabbit, where the viral DNA is partially methylated and some of it is integrated as long tandem repeats (Wettstein, University of California, Los Angeles).

The complete DNA sequences of BPV1 (7,945 base pairs; obtained by Chen, Levison and Seeburg of Genentech, and presented by Howley, National Cancer Institute, Bethesda) and of HPV1 (7,811 base pairs; Danos, Institut Pasteur) and the almost complete sequence of HPV6 (6,860 base pairs; Schwarz, University of Freiburg) were presented. In all cases, the viral genetic information is probably contained in only one strand, where four overlapping coding regions have been identified, as the other strand is blocked by stop codons in all three frames. The presumptive genes for both structural and non-structural proteins have been identified, and several putative splicing signals have been found in the non-structural region, strongly suggesting that appropriate splicing may generate a variety of transcripts.

The interpretation of the sequence data is corroborated by the mapping of the BPV1 transcripts. In productive warts (Howley; Ammann, Cancer Research Center, Heidelberg), RNA species are found which

*The workshop on 'Papillomaviruses and Cancer' was held in Freiburg, 7-8 April 1982, and was sponsored by the Deutsche Forschungsgemeinschaft.

M. Saveria Campo is at The Beatson Institute for Cancer Research, Garscube Estate, Switchback Road, Glasgow G61 1BD.

are absent in non-productive transformed cells and which only hybridize to the 30 per cent DNA fragment that is not necessary for cell transformation and contains the putative structural genes. These RNAs can be translated *in vitro*, and their polypeptide products appear to be genuine capsid proteins (Howley).

Four viral RNA species have been identified in BPV1-transformed cells, which, not surprisingly, hybridize to the 70 per cent DNA fragment capable of cellular transformation and thought to encode the non-structural viral antigens (Amtmann; Freese, University of Freiburg). More surprisingly, they are all co-terminal at their 3' end, but extend for different lengths to the 5' end where a 5'-leader sequence has been found (Amtmann). Thus, during the genesis of the non-structural BPV RNAs,

the same 5'-leader sequence would be spliced onto different bodies, to give rise to a family of several distinct transcripts, as predicted from the sequence data.

By the end of the workshop it had become clear that, if the progress of our knowledge of papillomaviruses continues at the same break-neck speed of the past few years, we can soon expect to learn about their mechanisms for interaction with, and transformation of, the host cell. □

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2. Jarrett, W. *et al.* *Nature* **274**, 215 (1978).
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4. Evans, I.A. in *Naturally Occurring Chemical Carcinogens*, 10th int. Cancer Congress (1970).

It is presumed that this largely hydrophobic sequence serves to anchor the VSG to the membrane at some stage during its biosynthesis, although it is certainly absent from mature VSGs. One possibility is that VSG gene activation involves the generation of a functional end, but DNA sequencing has shown that the codons specifying the hydrophobic tail are present in the basic copy genes⁹. The recombination event at the 3' end of the gene that leads to the generation of an ELC probably takes place just outside the coding region. In contrast, comparison of restriction enzyme sites around basic copy and ELC genes shows that a 1–2 kilobase (kb) 5' sequence is transposed with the gene in the generation of the ELC¹⁰. It is not known whether this sequence contains the promoter sequences allowing VSG expression when placed in transcriptionally active chromatin, or whether such promoters are present only at the expression locus. Nor is it known at what stage in the cell cycle of the bloodstream trypomastigote this duplication/transposition occurs.

So much is clear. What is in dispute is whether activation of VSG genes can occur only by the generation of an ELC. In particular, a group working at the International Laboratory for Research on Animal Diseases (ILRAD), Kenya, has consistently claimed that expression need not be linked to a specific duplication-transposition event. Thus an early analysis of one VSG gene was consistent with the presence of two gene copies, irrespective of whether that gene was being expressed or not^{11,12}. Sequence rearrangements around the two copies were observed in all trypanosome clones, even in different clones expressing the same antigen, and restriction enzyme mapping was compatible with the insertion and deletion of lengths of DNA between the 3' end of the cloned cDNA sequence and a group of restriction enzyme sites some 5–15 kb downstream from the end of the gene. No alterations in sequence were observed within at least 1–5 kb upstream from the 5' end of the cDNA sequence used as a probe. These results have now been extended to an analysis of VSG gene rearrangements in three different variants, A, B and C, all related by descent from a common, single trypanosome (see Majiwa *et al.* *Nature* **297**, 514; 1982), and surprisingly, each of the three variant antigen genes behaves differently. Probes specific for variant A recognize the same single fragment in Southern blots of total genome digests from variants A, B and C when using restriction enzymes which do not cut the cDNA, but also recognize an additional, larger fragment in DNA from the clone expressing VSG A. Such results are fully consistent with the ELC model. In contrast, as in the earlier studies, probes specific for VSG B recognize two fragments in the DNA from cells expressing VSGs A, B or C, but the size of

Antigenic variation in the trypanosome

from Mervyn Turner

PARASITES have evolved many methods of evading the immune system of their host¹, but perhaps none is so spectacular as the mechanism of antigenic variation adopted by the salivarian trypanosomes. These tsetse fly-transmitted parasites inhabit the bloodstream and tissue spaces of their mammalian hosts, where they rapidly stimulate the production of trypanocidal antibody. Before all the trypanosomes can be eliminated, however, antigenically distinct variants appear in the bloodstream which perpetuate the infection until they in turn are removed by antibody. Usually, the trypanosome can outstrip the immune system in this cycle, which leads eventually to the death of the host. How can such a simple eukaryotic organism produce sufficient antigenically distinct forms to subvert the sophisticated mechanisms possessed by the host for the generation of antibody diversity?

The antigenic profile of the trypanosome is governed by the structure of its surface coat, which in turn is made up of a densely packed layer of glycoprotein molecules, the variant surface glycoproteins (VSGs), whose composition is unique for each variant antigen type. The trypanosome genome contains in excess of 100 variant antigen genes probably generated by gene duplication and mutation², and by controlling the expression of these genes, the trypanosome is able to parade a sequence of different variants past the immune system of the host. Naturally, the mechanism by which expression is controlled has attracted much attention and a recent edition of *Nature* contains a contribution (Majiwa *et al.* **297**, 514; 1982) which is sure to add fuel to the controversy

surrounding the subject. Furthermore, a second contribution this week (Rice-Ficht *et al.*, p. 676) shows how the trypanosome may have developed a mechanism to extend its repertoire of antigens, perhaps indefinitely.

It has been clear for some time that VSG genes are capable of undergoing rearrangements. These rearrangements do not generate new variants in a fashion analogous to that used by immunoglobulins, but they are detected as alterations in the sequences flanking the VSG gene. Thus, Hoeijmakers *et al.*³ showed that expression of some VSG genes is accompanied by the generation of an additional copy of that gene, the expression-linked copy (ELC), in a different chromosomal location. Similar findings have been reported from other laboratories⁴, and Pays *et al.*⁵, by comparing the sensitivity to DNase I of both the basic copy and ELC of a VSG gene, concluded that the ELC was in transcriptionally active chromatin. To date, it has not proved possible to clone the ELC, but on the basis of R-looping, S₁ nuclease protection experiments and comparison of restriction enzyme sites in cDNA and in the ELC it has become apparent that VSG gene activation involves a duplication-transposition mechanism in which the 3' end of the gene is replaced in the formation of the ELC⁶.

Sequencing of cDNA clones has revealed that the 3' region of VSG mRNA encodes a 17–23 amino acid C-terminal extension^{7,8}.

Mervyn Turner is at the University of Cambridge Moltano Institute, Downing Street, Cambridge CB2 3EE.

both fragments differs in each variant. Has B lost its basic copy gene? This seems unlikely, because B was generated from A, which should therefore contain the basic copy of gene B. Is one of the B genes in an environment such that it is susceptible to the action of DNase I? Are both copies truly in chromosomal DNA? Answers to both questions are unknown. There appear to be four copies of gene C, in clones A, B and C. Two copies are invariant, whereas the sizes of the fragments carrying the other two genes alters in a way similar to that observed for variant antigen B.

Such results imply that different mechanisms may exist to control expression of VSG genes. The ELC model is attractive because it offers a ready explanation for control of antigenic variation, but as the principal proponents of the model themselves admit^{2,3}, not all the data are consistent with generation of an ELC. Another problem relates to the ploidy of trypanosomes. It has been estimated that ELC genes and basic copy genes exist in a ratio of 1:1 (ref. 4). If this estimate is correct, and if trypanosomes are diploid as generally assumed¹³, this implies coordinate expression of the products of two alleles. If the estimate is incorrect, some mechanism of allelic exclusion must operate. Such problems are not resolved by incorporating the results of the ILRAD group for their findings suggest no obvious mechanism for the control of antigenic variation. The two B-specific genes identified could, for example, be allelic forms of the same antigen, but it is not apparent how expression of each is controlled. Is one copy silent, and can the other be activated? Hence the importance of the DNase I protection experiment. Perhaps a very large fragment of DNA (>50 kb) is transposed into a site allowing expression. Such a phenomenon could be identified only by using restriction enzymes which cut outside the transposed unit and a detection system which allowed resolution of very large fragments. All groups agree that the sequence on the 3' side of the active gene is unusual^{2,12} in that it is apparently devoid of restriction enzyme sites for up to 15 kb, and is therefore presumably a simple repetitive sequence. The apparent clustering of many restriction enzyme sites at the end of this sequence could represent the end of the DNA molecule. Resolution of these paradoxes is awaited with interest.

As a further sophistication, VSG genes appear to be capable of localized 'somatic' hypermutation which may extend the antigenic diversity yet further. Sequence analyses of VSGs have shown that the N-terminal 350–400 amino acids are highly variable. The next 100 or so amino acids form a homology region which can be used to divide VSGs into two subsets, followed by the hydrophobic tail of about 20 amino acids which is absent from mature VSGs^{7,8,14–16}. Rice-Ficht *et al.* (see p.676) have now compared the DNA sequence of the basic copy gene with cDNA sequences



100 years ago

We observe that a correspondent of a daily paper proposes that men addicted to the pursuits of science should be called *scientiates*, after the Italian *scienziati*; and in like manner the studies of science, *sciential* studies (*studj scientzial*). The substitution of the American *scientist* for our unsatisfactory phrase *men of science* is of course much to be deprecated; perhaps we shall come to accept Sir William Thomson's proposed use of *naturalist* for the designation in question, if its sense may be extended. *Scientific studies* is a phrase which cannot be commended for accuracy.

A curious and little-known experiment, showing the resistance of the air in guns, is

described by Prof. Daniel Colladon, of Geneva. It resembles a feat that was sometimes performed by soldiers with the old Swiss carbines. M. Colladon fully charged with compressed air the hollow iron breech of an air-gun, serving as reservoir. Having screwed up the gun, he introduced a round lead ball, running freely, but nearly filling the bore; then, placing the gun vertical, he seized the upper end and pressed his thumb vigorously on the mouth. The gun was then "fired" by an assistant; the thumb remained in position, and the ball was heard to fall back in the bore. Thereupon, after recharging the breech and with the same ball, he shot the latter at a pine board about 4in. thick and it passed through. The experiment, M. Colladon says, is without danger, if the operator is sure of the strength of his thumb, if the gun is more than 32in. long, and if the ball is spherical and nearly fills the gun (in which it must act like a piston). While M. Colladon has repeated the experiment twenty or thirty times, without the least inconvenience a trial of it is perhaps hardly to be recommended.

From *Nature* 26, 353; August 10, 1882.

corresponding to the mRNA transcribed from the ELC of variant antigen gene A described by Majiwa *et al.* Surprisingly, considerable differences were observed in the variable region and in the C-terminal hydrophobic tail, amounting to nucleotide changes at four per cent of all codon positions. The homology region is identical in the two sequences. As previously recorded, the 3'-non-translated sequences are quite different in the two clones, consistent with generation of the ELC through recombination in this region. What could be the significance of such findings?

One possibility, which cannot be rigorously excluded, is that the 'wrong' basic copy gene has been cloned and sequenced. A second is that cDNA cloning artefacts are responsible. However, sequences from different cDNA clones were compared and found to be identical, and cloning artefacts are unlikely to respect homology regions. The assumption must therefore be that such sequence alterations are generated in the formation of the ELC, with the possibilities for 'somatic' diversity that that implies. Changes at nine per cent of codon positions in the influenza haemagglutinin are sufficient to produce new variants, corresponding to alterations at four antigenic determinants¹⁷, hence the trypanosome may be using this mechanism to extend enormously its repertoire of VSGs.

Two difficulties remain. First, only half the VSG variable region has so far been analysed in this way. Does the N-terminal region show similar hypermutation? Second, how much of this variable region is exposed on the trypanosome? It has been known for some time that immunizations with either living trypanosomes, or pure VSG can lead to the production of a high proportion of variant-specific monoclonal antibodies which will react with soluble VSG, but not with the surface of the

trypanosome^{18,19}.

Trypanosomes readily shed their VSG during infection, so much of the immune response must be against soluble VSG, but why have variant-specific determinants on soluble VSGs which are not exposed on the surface of the trypanosome, and which are therefore not under selective pressure? Of the several potential antigenic determinants on the VSG molecule only a small number may be exposed on the closely packed surface coat, and such sites may be 'hot spots' for the 'somatic' hypermutation described by Rice-Ficht *et al.* For the trypanosome to then induce the host to make variant-specific antibody which cannot recognize the trypanosome surface could be a diversionary form of antigenic competition which surely would be the ultimate caprice of this virtuoso parasite? □

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Anilides and the Spanish toxic oil syndrome

from A. Pestaña and E. Muñoz

DURING May and June, 1981, an epidemic outbreak of interstitial pneumonitis occurred in Spain. Over 20,000 people were affected, and nearly five per cent subsequently developed persistent neuro-dystrophic- and scleroderma-like conditions. More than 300 people have died. The disease, at first attributed to a never isolated mycoplasma, is now thought to be due to the consumption of adulterated cooking oil containing the processed products of crude rapeseed oil denatured with 2 per cent aniline. The oil was enriched in fatty acid anilides (up to 2,000 p.p.m.) and almost freed from aniline (less than 30 p.p.m.), as a result of thermal processing (220°C) under vacuum.

Comprehensive research into the causes of the toxic oil syndrome was slow to start but tremendous efforts are now being made by many clinical and research institutes — although lack of effective co-ordination still remains a problem. Progress made so far was reviewed at a National Symposium¹ organized by the Ministry of Health and held in Madrid on 11–12 June.

Initial doubts about the toxicity of anilides and uncertainties in the cause-effect relationships between the disease and the available samples of suspicious oils led the Consejo Superior Investigaciones Científicas (CSIC) to a research programme based on two priorities. First, a series of screening tests of a wide sample of oils was to be carried out in HeLa cells to uncover toxic factors. Second, a comparative study of synthetic anilides and suspicious oils would be made in several animal models, such as chick embryo, mouse, rat and rabbits. After 9 months of intensive research, a working hypothesis of the toxic oil syndrome can be put forward in light of the clinical findings reported at the meeting.

Toxicological screening of more than 100 samples of oil led the CSIC researchers to conclude that the *in vitro* test is probably not directly relevant to human and animal pathology. No correlation was found between cell toxicity and anilide content of the oils, and the synthetic anilides were found to be almost devoid of *in vitro* toxicity. The direct toxicity of the oils seems to depend on their content of free linoleic acid and/or its oxidation products, such as hydroxy and epoxy derivatives.

Most of the suspicious oils were found to be a mixture of vegetable oils (rapeseed, olive seeds and grapeseeds) and liquid fats, extracted from lard. Their average linoleic

acid content was high (42 per cent of the total fatty acid) and their content of lipid peroxides was also very high (four times higher than the legal limits, on average). Furthermore, all the oil samples were very susceptible to oxidative damage, indicating a low content of natural antioxidants, the tocopherols.

As it is generally thought that the tocopherols (vitamin E compounds) mainly function as antioxidants for unsaturated lipids and so protect lipid membranes from attack by free radicals, it is possible that consumers of the oil mixtures may have developed vitamin E deficiency. They would have been subjected both to a higher requirement for vitamin E (because of the oil's high linoleic and peroxide contents) and to a decreased dietary supply (because of the oil's lack of tocopherols).

We do not, however, believe that this would suffice to explain the epidemic outbreak of the toxic oil syndrome as suggested by McMurray², since it is generally accepted that similar mixtures (devoid of anilides) have been fraudulently marketed for years. But we think that it must be considered an important pathogenic factor, in association with fatty acid anilides or their presumed imino-quinone derivatives, as has been shown with *p*-hydroxy acetanilides³.

There are no clinical data in support of free radical pathology. A systematic study of the vitamin E content in humans affected by the toxic oil syndrome is also lacking. However, an increase in the product of peroxidative damage of membrane lipids (assayable with thiobarbituric acid) has been reported in human necropsies and experimental animals after anilide treatment. A marked decrease in T-cell immunosuppressive functions in toxic oil syndrome patients, which is an expected result of free-radical pathology⁴ and vitamin E deficiency⁵, was also reported.

Working with chicken embryo, García-Gancedo *et al.* have shown that the toxic effects of synthetic anilides can be prevented by the simultaneous administration of tocopherol. The result indicates that the anilides are acting, at least in this experimental model, through a toxic route susceptible to free radical scavengers. Clinical trials with vitamin E were ineffective, but it should be remembered that the disease was at an advanced stage when the treatment was applied.

The experiments employing chicken embryo, carried out by the CSIC, deserve a few comments. First, the toxicity of anilides is dose related. Second, toxicity appears only 2–3 days after hatching and does not affect embryonic development, suggesting the involvement of inhaled oxygen on the toxicity of anilides. Last, but not least, the toxic syndrome in chicken evolves rapidly towards respiratory distress

and paralysis. Histopathological study of the affected animals reveals a perivascular infiltrate with eosinophils, interstitial pneumonitis with abundant eosinophils in the alveolar lumen and widespread lesions in the cerebellum, with pyknotic degeneration of the Purkinje cells and vacuolar degeneration of the white matter. These same pathological signs were found in rabbits treated with low doses of synthetic anilides.

Work with rabbits was aimed at elucidating the involvement of immunological mechanism in the toxicity of anilides — a possibility that arises from the clinical signs (fever, rash with marked eosinophilia and large titres of IgE) and the structural relationships between anilides and penta-decil cathecol, the agent of the delayed hypersensitivity to poison ivy⁶. Rabbits treated for 15 days with oral anilides (10 µg per kg) showed a typical delayed response in a skin test with anilides and titratable antibodies against the same synthetic anilides. Moreover, anilide-treated animals developed a syndrome of progressive paralysis and the cerebellar lesions (Purkinje cells and white matter) described above, in close resemblance to the Gordon phenomenon⁷. In addition, eosinophilic infiltrates were seen in the skin lesions after challenge with anilides and in the pia madre around the cerebellar cortex.

Some results from human necropsies indicate the occurrence of similar lesions in human brains. There is also histochemical and electron microscopic evidence (Rycol, personal communication) of peroxidase-positive granula in the vicinity of the neural lesions observed in muscle biopsies from toxic oil patients.

On the basis of these clinical and experimental observations, we think that the pathogenesis of the toxic oil syndrome could be explained by an eosinophil-mediated cytotoxicity, probably directed by specific antibodies towards anilides, that could be incorporated in the cellular plasma membranes⁸. These immunopathological causative effects (relatively well documented in toxic oil syndrome humans with depression in T-cell suppressor functions, eosinophilia, high IgE levels and autoantibodies) could have arisen through the synergistic effects of anilides and the peroxidative status, resulting from the poor nutritional quality of the oil mixtures. Although we do not exclude other pathogenic possibilities, we think that these ideas may provide a conceptual framework and working hypothesis by which the Spanish toxic oil syndrome could be experimentally approached. □

A. Pestaña, Instituto de Enzimología, CSIC, is responsible for the coordination of the CSIC programme on toxic oil syndrome and E. Muñoz, Unidad de Biomembranas, Centro de Investigaciones Biológicas, is Vicepresident for Scientific Affairs at the CSIC.

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ARTICLES

Aspherical Earth structure from fundamental spheroidal-mode data

Guy Masters, Thomas H. Jordan, Paul G. Silver & Freeman Gilbert

Institute of Geophysics and Planetary Physics, University of California, San Diego, La Jolla, California 92093, USA

Free-oscillation data reveal heterogeneity in the Earth's mantle whose geographical pattern is dominated by spherical harmonics of angular degree two and correlates well with the hydrostatically referenced geoid. The heterogeneity can be modelled as localized in the transition zone (420–670 km depth) and may be related to a large-scale component of mantle convection.

THE study of the Earth's lateral, or aspherical, heterogeneity using long-period seismic observations has since 1966¹ been based on the 'pure-path' approximation; variations in the phase velocities of travelling waves are represented as a sum of their fractional dispersion across several types of tectonic structures. Recently, this sort of analysis has been extended to normal-mode frequency shifts². We investigate here a new data set of nearly 4,000 fundamental-mode frequencies measured from over 500 records of the International Deployment of Accelerometers (IDA) network. The extensive coverage provided by these measurements shows that the present schemes of tectonic regionalization are inappropriate at periods longer than ~200 s; the lateral heterogeneity revealed by the low-order fundamental modes is more suitably represented by a spherical-harmonic expansion with the dominant power in the degree-two terms. A drawback of this representation is that the data do not constrain the odd-order harmonics^{3,4}, a fact which must be kept in mind when interpreting the observed patterns. The source of the observed signal cannot be determined unambiguously, but our analysis suggests that the transition zone of the Earth's mantle (between about 400 and 700 km depth) is the most plausible location of the dominant heterogeneity.

Observations of fundamental-mode frequencies

In a recent study of fundamental-mode attenuation the complex frequencies of the modes ${}_0S_8$ to ${}_0S_{46}$ from 557 IDA recordings were measured⁵. Because the primary goal was to obtain reliable Q determinations, only large-amplitude peaks showing little signs of overtone interference or beating due to splitting were retained. A by-product of this analysis was 3,934 reliable measurements of fundamental-mode frequencies, forming the data base for the present study. Below $l = 39$ each mode has an average of 118 independent observations, but the number of measurements decreases rapidly at higher angular orders. The modes above $l = 43$ had <20 observations and were dropped from the data set. A complete account of the data analysis techniques can be found in ref. 5.

A convenient way of displaying the data is to plot the observed frequency shifts at the location of the pole of the great circle joining the source and receiver. In the geometrical optics limit, when the horizontal wavelength of the heterogeneity is much greater than that of the mode, the frequency shift is solely a function of the pole position⁶. If this condition is not met in the Earth, then effects not described by this asymptotic theory become important, and a more complete interpretation may be required^{6,7}. In particular, the exact first-order expressions for the frequency shifts depend on the details of the source mechanisms, so that if non-asymptotic effects

were to dominate the signal, the apparent frequencies would not be a smooth function of pole position. Figure 1 shows the frequency shifts for several sets of angular orders. The patterns revealed by these diagrams have a large-scale simplicity and are consistent from angular order to angular order; this suggests that the geometrical optics limit is a valid approximation and that the asymptotic theory can provide an adequate framework for the interpretation of the data.

Theoretical developments^{6,8} define the peak location in terms of the ratio of the first polynomial moment of the multiplet spectrum to its zeroth moment, an estimator employed in the first systematic observational study². The values used here were derived by least-squares fitting of a singlet shape to the observed spectrum⁵. However, for the large-amplitude, singlet-like peaks considered in the present study, these different estimators give almost identical results, a behaviour consistent with the asymptotic theory⁸. The least-squares estimation technique is less sensitive to contamination by nearby higher modes and has the additional benefit of a comprehensive error analysis⁹. The errors in the frequency estimates are typically 0.05%, roughly a factor of 10 less than the frequency variations ascribed to aspherical heterogeneity.

One well determined feature of the Earth's asphericity is its ellipticity of figure, which causes a positive frequency shift for poles at low latitudes and a negative shift at high latitudes. The expected variation with latitude—amounting to 2.2 μHz for ${}_0S_8$ and 9.3 μHz for ${}_0S_{43}$ —is evident at low angular orders, but above $l = 16$ or so it disappears almost completely. This behaviour has been attributed by Silver and Jordan² to the existence of some sort of lateral heterogeneity which, in terms of its effects on the fundamental spheroidal modes, is anti-correlated with the ellipticity of figure. The present study confirms this conclusion. Because we believe the hydrostatic component of ellipticity is well modelled by the Clairaut theory, we have adopted an elliptical Earth as a reference model and have corrected the raw peak frequencies for this signal using the asymptotic formula derived by Dahlen¹⁰.

Table 1 Localized models of degree-two aspherical heterogeneity

Zone	Amplitude*	δm_2^0	Normalized coefficients				Total variance reduction (%)
			$\text{Re } \delta m_2^1$	$\text{Im } \delta m_2^1$	$\text{Re } \delta m_2^2$	$\text{Im } \delta m_2^2$	
1	1.84	-0.70	0.27	-0.17	0.12	0.62	52
2	1.76	-0.72	0.26	-0.15	0.20	0.59	61
3	2.10	-0.69	0.25	-0.13	0.27	0.60	49
4	7.74	-0.67	0.26	-0.13	0.26	0.63	46
5	10.62	-0.70	0.26	-0.16	0.19	0.61	59

* Amplitude of the heterogeneity as a percentage of shear velocity; perturbations in density and compressional velocity are constrained to have relative amplitudes of 0.4 and 0.8 of the shear-velocity perturbation (see text).

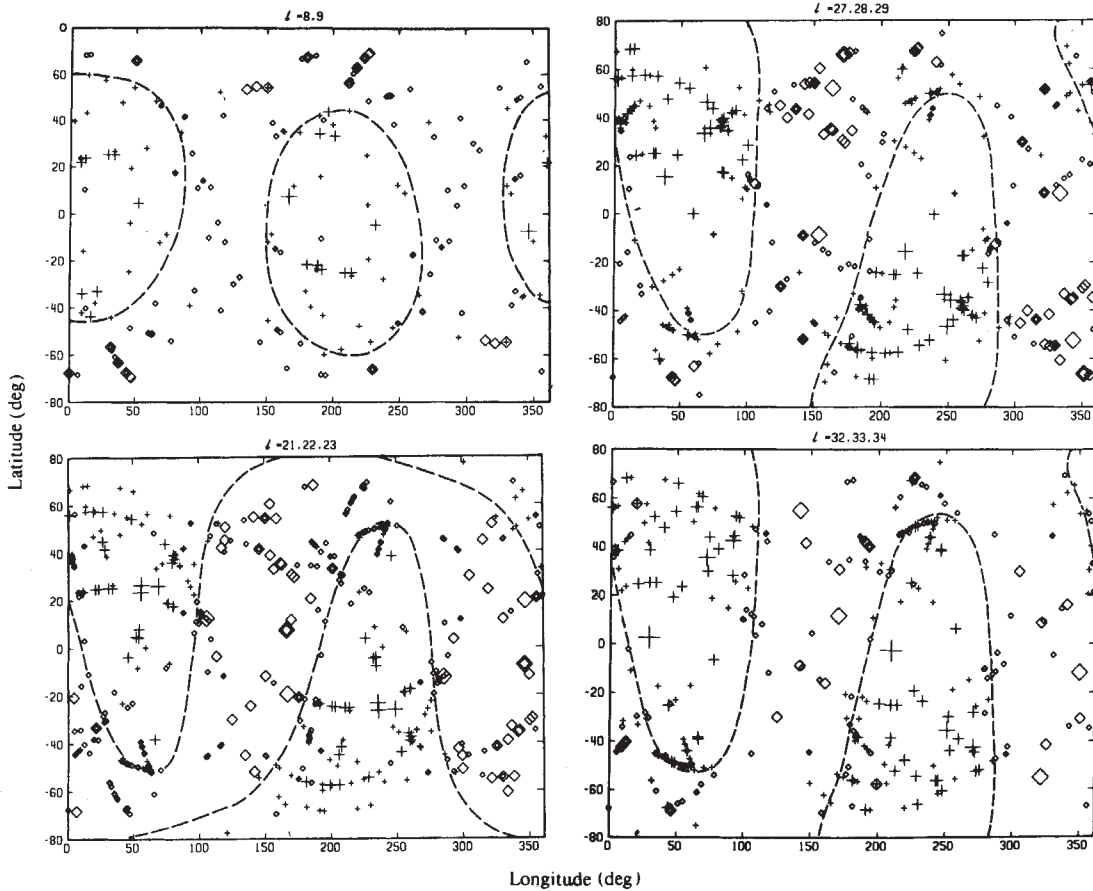


Fig. 1 Multiplet-location data from selected fundamental spheroidal modes plotted at the poles of the great circles connecting the sources and receivers. Frequency shifts are relative to the estimated degenerate eigenfrequencies of Fig. 2c. Symbol type indicates sign of the frequency shift (+, positive; $\diamond$, negative); symbol size indicates its magnitude (smallest symbols correspond to shifts of 0.0–0.1%; the largest to 0.3–0.4%). Dashed lines are the nodes of the best-fitting degree-two patterns for $\delta\omega_{\text{local}}$ uncorrected for ellipticity of figure; the patterns are very similar except for the P_2^0 components, which are large for $l = 8, 9$ but small for the other modes shown.

Models of the heterogeneity

The elastic part of the terrestrial monopole is specified by $\bar{\mathbf{m}}(r) = [\bar{\rho}(r), \bar{v}_p(r), \bar{v}_s(r)]$, where r is radius ($0 \leq r \leq a = 6,371$ km) and $\bar{\rho}$, $\bar{v}_p$ and $\bar{v}_s$ are the spherically averaged mass density, compressional velocity and shear velocity, respectively. The degenerate eigenfrequency of the k th mode of this spherically symmetric model is denoted $\bar{\omega}_k$. Aspherical heterogeneity is represented by the perturbation

$$\delta\mathbf{m}(r, \theta, \phi) = \sum_{s=1}^{\infty} \sum_{t=-s}^s \delta\mathbf{m}_s^{(t)}(r) Y_s^{(t)}(\theta, \phi) \quad (1)$$

where $Y_s^{(t)}$ is the complex surface spherical harmonic of degree s and order t ; the conventions of Edmonds¹¹ are adopted, so that $Y_s^{(-t)} = (-1)^t Y_s^{(t)*}$. Because the perturbation is real, $\delta\mathbf{m}_s^{(-t)} = (-1)^t \delta\mathbf{m}_s^{(t)*}$.

The asymptotic first-order theory relating the multiplet locations to the aspherical model (1) is well developed^{6,8}; for a source and receiver connected by a great circle with pole at colatitude Θ and longitude Φ , the peak frequency of a mode of radial order n and angular order $l \gg n$ is

$$\omega_k = \bar{\omega}_k + \sum_{s=2}^{\infty} P_s(0) \sum_{t=-s}^s (\delta\omega_{\text{local}}^{(k)})_s^t Y_s^{(t)}(\Theta, \Phi) \quad (2)$$

The coefficients in this expansion are given by

$$(\delta\omega_{\text{local}}^{(k)})_s^t = \int_0^a \mathbf{M}_0^{(k)}(r) \cdot \delta\mathbf{m}_s^{(t)}(r) r^2 dr \quad (3)$$

where $\mathbf{M}_0^{(k)}$ is a triplet of functional derivatives computable from the eigenfunctions of the terrestrial monopole¹². The apparent eigenfrequency (2) can be shown to equal the great-circular average of the 'local eigenfrequency' $\bar{\omega}_k + \delta\omega_{\text{local}}^{(k)}(\theta, \phi)$,

whose harmonic coefficients are defined by equation (3). Such averages place no constraints whatsoever on the odd-parity (s -odd) part of the aspherical perturbation, a point first noted by Backus³ in his analysis of great-circle dispersion. Equations (2) and (3) can be combined to give a linear inverse problem for the even part of $\delta\mathbf{m}$.

Alternatively, equation (2) can be solved directly for $\delta\omega_{\text{local}}^{(k)}$, permitting an investigation of the even part of the spherical harmonic patterns underlying individual modes without introducing the additional questions of nonuniqueness associated with the complete inverse problem. Figure 2 shows the results of this analysis assuming $\delta\omega_{\text{local}}^{(k)}$ is representable by harmonics of degree two. To reduce the variance and ensure a good sampling of the sphere, the data from three consecutive angular orders were combined in each calculation. The inversion determined estimates of the degenerate eigenfrequencies and the complex coefficients of $s=2$, for a total of eight independent parameters per mode triplet. The coefficients in Fig. 2a have been normalized to unit power to facilitate comparisons among the modes; their normalization scalars are shown in Fig. 2b.

The estimated degenerate eigenfrequencies are plotted relative to the PEM-A model¹³ in Fig. 2c, together with the weighted means of the apparent frequencies and the estimates given in ref. 2. (Figure 2c was constructed using the PEM-A eigenfrequencies published by Dziewonski *et al.*¹³; these differ from the values calculated from the PEM-A model by up to 2 μHz .) The offset in the eigenfrequencies between ${}_0S_{11}$ and ${}_0S_{18}$, is prominent. Present Earth models predict significant quasi-degenerate Coriolis coupling between the ${}_0S_l$ and ${}_0T_{l+1}$ modes at the ends of this band¹⁴, which could explain the shifts of ${}_0S_{11}$ and ${}_0S_{18}$, but the reason for a nearly constant offset of the

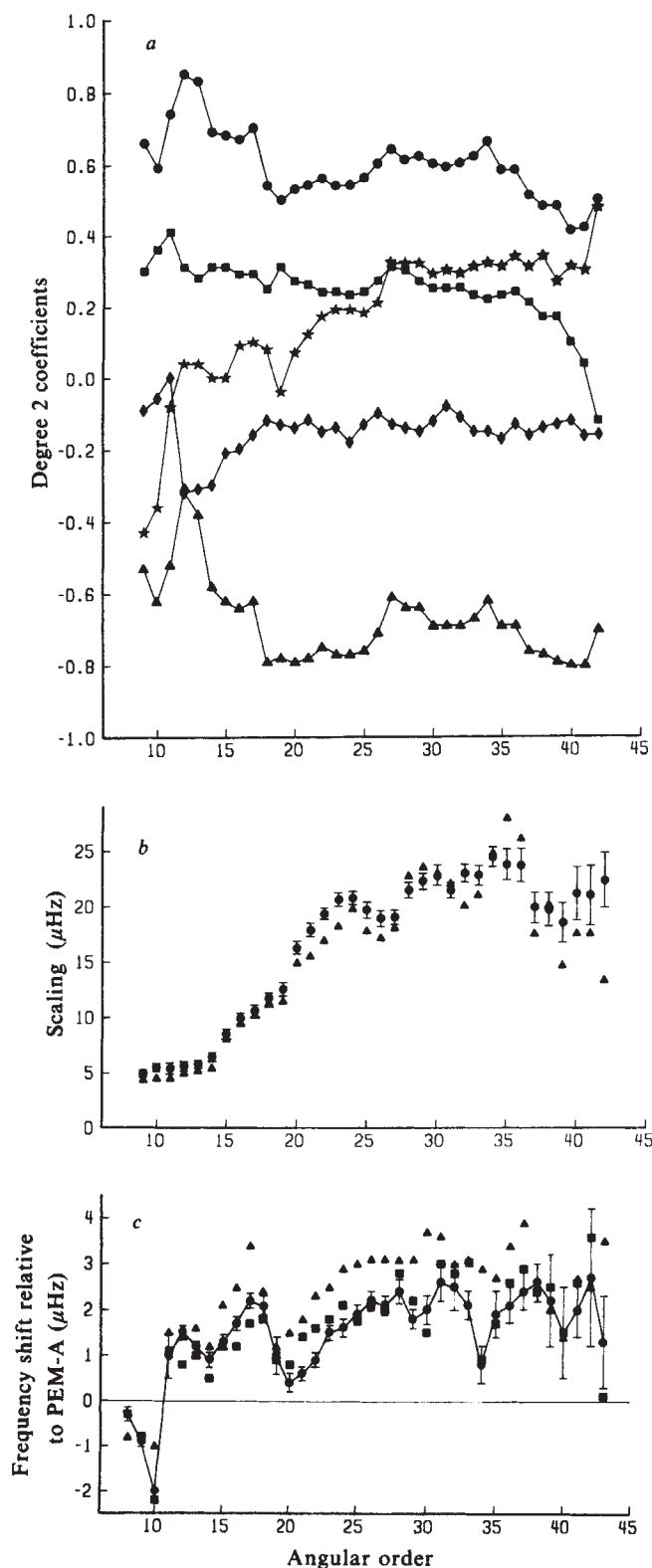


Fig. 2 *a*, Normalized degree-two coefficients of $\delta\omega_{\text{local}}$ from a least-squares fit to the ellipticity-corrected multiplet locations assuming $s_{\text{max}} = 2$ (Δ , P_2^0 ; $\blacksquare$, $\text{Re } P_2^1$; $\blacklozenge$, $\text{Im } P_2^1$; $\star$, $\text{Re } P_2^2$; $\bullet$, $\text{Im } P_2^2$). Three consecutive angular orders were combined in each calculation (eight-parameter inversion). *b*, Scale factors for these coefficients, with 1σ error bars. Δ , Degree-two scale factors for 30-parameter ($s_{\text{max}} = 6$) inversions. *c*, Estimates of the degenerate eigenfrequencies $\bar{\omega}_k$ plotted as residuals with respect to published eigenfrequencies of the PEM-A model¹³. Values from eight-parameter inversions are connected by a solid line; error bars are 1σ . Simple weighted means of our data ($\blacksquare$) and those from ref. 2 (Δ) are shown. The latter two estimates are more sensitive to bias caused by incomplete sampling of the aspherical heterogeneity.

intermediate modes remains obscure. This important observation, previously noted in ref. 2, may provide strong constraints on the spherically averaged Earth structure.

Note that the average eigenfrequencies estimated by the degree-two inversion form a generally smoother function of angular order than the simple weighted means, particularly at the higher angular orders. This is because the pole positions for our data set are biased to temperate latitudes and there consequently exists a significant tradeoff between $\bar{\omega}_k$ and any P_2^0 component of $\delta\omega_{\text{local}}^{(k)}$. At the higher angular orders the acceptable data are fewer, the sampling is more variable, and the P_2^0 component is large; hence, a simple averaging of the data can introduce spurious variations in estimates of $\bar{\omega}_k$. The small positive shift of the Silver–Jordan means relative to our estimates appears to be ascribable to a bias in the way their pole positions sample the patterns shown in Fig. 1.

Several conclusions can be immediately drawn from Fig. 2*a, b*. The normalized spherical harmonic pattern of $\delta\omega_{\text{local}}^{(k)}$ is quite stable, especially for the modes ${}_0S_{20}$ – ${}_0S_{38}$, where only the real part of the P_2^2 term appears to exhibit a significant trend. The pattern consists of a large P_2^2 component and a large negative P_2^0 component. It is this latter term which at the higher angular orders almost cancels the signal due to the ellipticity of figure. The P_2^1 component is comparatively small, accounting for only $\sim 10\%$ of the total power. The scale factors multiplying this nearly constant pattern increase with angular order to $l \sim 35$, but level off at higher angular orders.

The variance reduction provided by this simple model is remarkable (Fig. 3). Except for ${}_0S_{11}$ whose scatter is probably dominated by the vagaries of quasi-degenerate coupling, the median variance reduction is 60% and, in the centre of our mode band where the data are most numerous, it exceeds 70%.

This variance reduction is far superior to that achievable by tectonic regionalizations with comparable numbers of parameters. We have, for example, inverted these data using a six-parameter regionalization^{2,15}. The median variance reduction is only 24% and for only nine of the 35 modes does it exceed 30% (Fig. 3). Furthermore, the patterns in $\delta\omega_{\text{local}}^{(k)}$ derived from the tectonic regionalization are much less stable with respect to variations in angular order number.

The possibility that power is aliased into the degree-two coefficients from higher-degree heterogeneity by inadequate sampling of the sphere can be checked by comparing these results with inversions allowing higher harmonics in $\delta\omega_{\text{local}}^{(k)}$. The scale factors for the degree-two coefficients from inversions with $s_{\text{max}} = 6$ (30 parameters per mode triplet) are plotted with those from the eight-parameter inversions in Fig. 2*b*. Throughout most of the band the agreement is quite good. In fact, below $l = 36$ the power in the higher harmonics is down by a factor of four or more. Above $l = 36$ the scale factors for the 30-parameter inversion are generally lower, the power in the higher harmonics increases, and the possibility of appreciable aliasing cannot be discounted. As shown in Fig. 2*b*, ignoring higher harmonics tends to increase the degree-two scale factors for $l > 36$. Because this bias appears to be small and the variance reduction at the lower angular-order numbers is not significantly improved by the addition of the higher-degree terms, we shall restrict further attention to the degree-two components.

The uniformity of the $\delta\omega_{\text{local}}^{(k)}$ patterns suggests that the dominant signal arises from anomalies localized in radius. If this is true, the variation of the pattern's amplitude with angular order constrains the position of the heterogeneity inside the Earth. We consider five zones based on the major seismological divisions of the mantle as defined by the PEM-A model:

- Zone 1: 5,350–5,701 km (top of the lower mantle)
- Zone 2: 5,701–5,951 km (transition zone)
- Zone 3: 5,951–6,151 km
- Zone 4: 6,151–6,291 km (low-velocity zone)
- Zone 5: 6,291–6,352 km (lid)

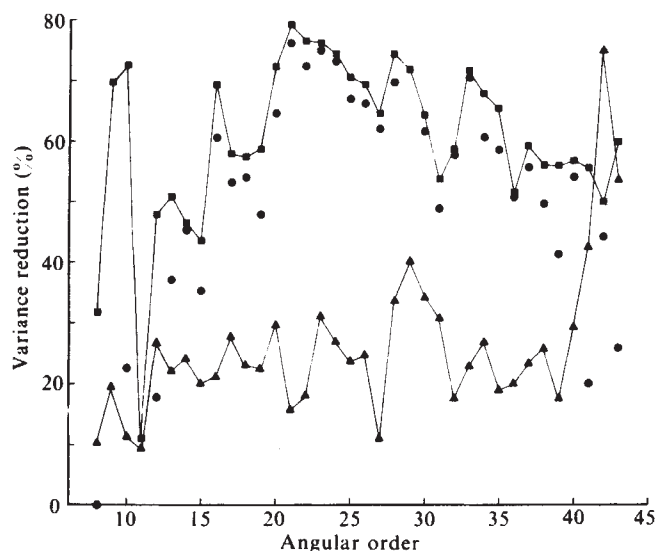


Fig. 3 Percentage variance reductions achieved by the degree-two representation of $\delta\omega_{\text{local}}^{(k)}$ given by Fig. 2 (—■—), the tectonic regionalization of Jordan¹⁵ (—▲—), and the degree-two transition-zone (zone 2) model of Table 1 (—●—).

Five models of degree-two heterogeneity have been constructed by least-squares inversion of the entire data set (excluding ω_{11}) assuming the perturbations to ρ , v_p and v_s are constant functions of radius localized in one of these zones. Of course, the radii of the discontinuities separating zones could be (and probably are) laterally variable, but their effects on the data have been shown by numerical experiments to be similar to constant perturbations in adjacent zones; for these preliminary experiments we have therefore fixed these radii.

If ρ , v_p and v_s are allowed to be independently variable in the inversion, then the results depend strongly on the integrals of the functional derivatives across each layer. In zones 4 and 5, for example, the differential kernels for ρ and v_p are of the same magnitude but opposite sign, and the inversion yields models with large positive ρ perturbations correlated with large negative v_p perturbations, and vice versa. From what we know about mantle properties this behaviour is unphysical. To eliminate such models we have scaled *a priori* the perturbations in density and compressional velocity to those in shear velocity by assuming $d \ln \rho / d \ln v_s = 0.4$ and $d \ln v_p / d \ln v_s = 0.8$. These scalings were derived from the temperature derivatives of possible mantle constituents given by Anderson *et al.*¹⁶, but they are equally appropriate for the chemical variations observed in naturally occurring periodotites^{17,18}. If the heterogeneity is confined to zones 1, 2 or 3, then the scaling is unimportant, as the modes are primarily sensitive to only the v_s perturbations in these regions. This is not true for zones 4 and 5, where the ρ and v_p perturbations predominate, although it is unlikely that any reasonable scaling will alter our conclusions.

The results of the inversions for the five zones are listed in Table 1. The spherical-harmonic pattern is essentially the same for each zone, but the magnitude of the requisite perturbation increases rapidly for the shallower zones. The localized perturbation giving the best overall fit to the data is the zone-2 (transition-zone) model with a total normalized variance reduction of 61%. Except at the very low and high ends of the mode band, the variance reduction provided by this simple model is almost as great as the optimal values for a degree-two parameterization attained by the $\delta\omega_{\text{local}}^{(k)}$ inversions (Fig. 3). Zones 1, 3 and 4 have much less satisfactory residuals. Although the zone-5 (lid) model is better, it systematically under-predicts the magnitude of the frequency shifts in the band $\omega_{20}-\omega_{30}$ and overpredicts the shifts above ω_{35} .

These aspects of the data fit are illustrated in Fig. 4, which compares the scaling of the degree-two pattern derived from the $\delta\omega_{\text{local}}^{(k)}$ inversions with the scalings predicted by the various models. The dependence of the scaling on angular-order number is clearly best matched by the transition-zone model. The zone-1 solution predicts too rapid a roll-off, whereas the models for zones above the 420-km discontinuity do not roll off fast enough.

Correlation with the geoid

The geographical pattern of aspherical heterogeneity synthesized from the transition-zone model of Table 1 is mapped in Fig. 5 (the contours for the other models are very similar). High-velocity anomalies are centred near the Equator in the western Pacific and central Atlantic, whereas lows occur at high latitudes in the southeastern Pacific and northern Eurasia. These features are remarkably similar to the dominant large-scale undulations of the geoid when the latter is referenced to the hydrostatic ellipsoid. The correlation coefficient between the degree-two harmonics of the transition-zone model and the GEM 9 geoid¹⁹ corrected for Jeffreys' hydrostatic value of J_2 (ref. 20) is +0.79, which is significant at the 94% confidence level. We hypothesize, therefore, that the heterogeneity detected by the multiplet-location data is responsible, at least in part, for the degree-two geoid anomalies.

None of the localized perturbations in Table 1 can by itself adequately account for these anomalies, however; the predicted amplitudes are too large. For example, the root-mean-square (r.m.s.) geoid amplitude calculated for the transition-zone

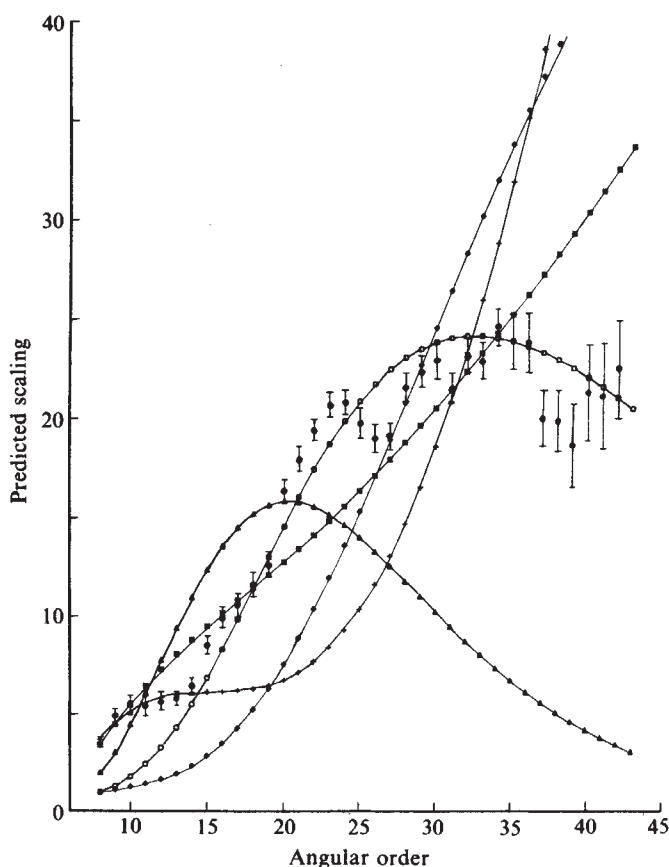


Fig. 4 Variation of degree-two scale factors with angular order predicted by localized models of aspherical heterogeneity (Δ , zone 1; $\square$, zone 2; $\diamond$, zone 3; $+$, zone 4; $*$, zone 5). Solid circles with 1σ error bars are the degree-two scalings from the eight-parameter inversions shown in Fig. 2b.

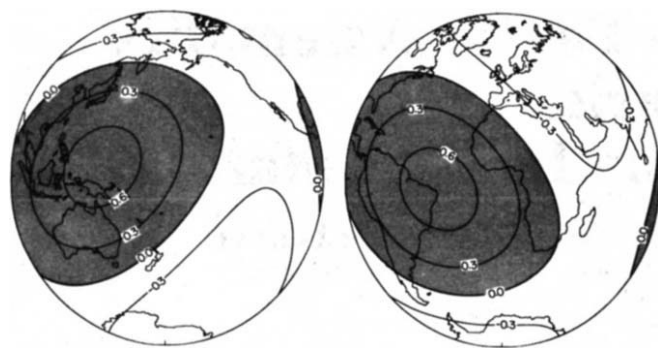


Fig. 5 Lambert equal-area projections of the Earth showing the degree-two heterogeneity of the transition-zone model, normalized such that its square integrates to unity on the unit sphere. Regions of positive anomaly are shaded; contour interval is 0.3 units.

models exceeds the observed degree-two value by about a factor of five. The discrepancy for the zone-5 model is even larger, nearly a factor of nine. Either the velocity-density scaling adopted in the inversions is grossly incorrect or the heterogeneity sensed by the multiplet-location data is compensated at other levels within the Earth. We prefer the latter hypothesis, as the former would imply substantial lateral variations in mean atomic weight¹⁷ which would be difficult to maintain in a convecting mantle.

The compensation is not likely to be confined to the upper mantle. If our scaling parameters are roughly correct, then only models with large and nearly cancelling radial excursions in upper-mantle properties can satisfy both the multiplet-location data and the geoid. It seems improbable that any mechanism responsible for such excursions would yield multiplet-location patterns so similar at different angular orders. A lower-mantle compensation is more plausible. The changes in the $\delta\omega_{\text{local}}^{(k)}$ patterns at low angular orders are suggestive of a deeper-seated heterogeneity, as are the observations of splitting of high- Q , low-angular-order modes²¹. In fact, heterogeneities anti-correlated with the geoid have already been proposed for depths greater than 1,100 km by Dziewonski *et al.*²² to explain certain large-scale variations in P -wave travel times.

Discussion

Aspherical heterogeneity of the sort implied by the transition-zone model could be produced by lateral temperature differences of the order of 200–300 °C associated with a large-scale component of mantle convection. Such variations could occur in a thermal boundary layer between the upper and lower mantle, or alternatively, in a mantle-wide system of convective flow. To differentiate between these possibilities will require better resolution of the heterogeneity distribution as a function of depth. Although the simple scaling of the $\delta\omega_{\text{local}}^{(k)}$ patterns with angular order suggests some localization in depth and motivates the modelling experiments of Table 1, the data now

in hand certainly do not exclude models wherein the aspherical variations are distributed throughout the Earth. Indeed, we have argued that the geoid requires the heterogeneity constrained by the multiplet-location data to be largely compensated elsewhere in the mantle, most likely near its base.

There are at least four additional sources of seismic data which should help to increase our vertical resolution of structure. The first is the measurement of phase-velocity dispersion at periods shorter than 200s. We have attempted to extend our data set using published phase-velocity observations, but these have proved inadequate in both quantity and quality to improve our spherical-harmonic models of lateral heterogeneity. The second is the study of fundamental toroidal modes whose energy is more strongly concentrated near the surface than spheroidal modes, and the third is the measurement of resolvably split high- Q modes of low angular order. Finally, the inversion of large sets of teleseismic travel times²² can be used to constrain the odd-order harmonics of deep-seated heterogeneity to which the free-oscillation data are insensitive. With the addition of these data detailed models of the Earth's large-scale lateral heterogeneity should be within our reach in the near future.

Conclusions

A data set comprising 3,886 apparent centre frequencies of the modes ${}_0S_8$ – ${}_0S_{43}$ measured from over 500 IDA records has been analysed for aspherical Earth structure. When plotted as a function of the pole position of the great circle connecting the source and receiver the frequency shifts show a remarkably coherent geographical pattern clearly dominated by surface spherical harmonics of degree two. Although the degree-two pattern is well constrained by the data, the depth distribution of the aspherical heterogeneity cannot be determined unambiguously. Models of degree-two heterogeneity localized in various mantle layers have been constructed using the asymptotic theory of multiplet locations by scaling the density and compressional-velocity perturbations to those in shear velocity. The best fit to the data is obtained by localizing the degree-two perturbations in the mantle transition zone (420–670 km depth). This transition-zone model is characterized by shear-velocity variations of 1.7% (r.m.s. amplitude). The overall variance reduction provided by the model is 61%, more than a factor of two better than that achievable by tectonic regionalization. The degree-two pattern has high-velocity anomalies centred near the Equator in the western Pacific and central Atlantic and lows at high latitudes in the southeastern Pacific and northern Eurasia; these features correlate well with the hydrostatically referenced geoid. The transition-zone model predicts geoidal undulations too large by about a factor of five, however, suggesting a compensation mechanism which we infer to be heterogeneity in the lower mantle. Aspherical heterogeneity of the sort implied by our data could be produced by lateral temperature differences of the order of 200–300 °C associated with a large-scale component of mantle convection.

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Sm–Nd, Rb–Sr and U–Th–Pb systematics of granulite facies rocks from Fyfe Hills, Enderby Land, Antarctica

D. J. DePaolo*, W. I. Manton†, E. S. Grew* & Martin Halpern††

* Department of Earth and Space Sciences, University of California, Los Angeles, California 90024, USA

† Geosciences Programs, University of Texas at Dallas, Richardson, Texas 75080, USA

‡ Present address: Enserch Exploration, Inc., Dallas, Texas 75221, USA

New Sm–Nd isotopic measurements made on granulite-facies metamorphic rocks from East Antarctica provide firm evidence that crust of ~3,500-Myr age exists in the Fyfe Hills. Zircon U–Pb data provide further documentation for a granulite-facies event 2,500 Myr ago, during which Rb, U, Sm and Nd were highly mobile. The U–Pb and Rb–Sr isotopic systems were seriously disrupted and in most samples give meaningless model ages. In contrast, Sm–Nd model ages were offset only slightly. A large time interval of 1,000 Myr between the times of crust formation and granulite-facies metamorphism has not previously been reported for Archaean rocks, and suggests that 2,500 Myr ago the Fyfe Hills may have been located in an Andean- or Himalayan-type continental margin where crust already 1,000-Myr old was involved in an orogenic event.

THE Napier complex of coastal Enderby Land, East Antarctica (46°–57°E) (Fig. 1) is an Archaean granulite-facies terrane^{1–3} that was cut by pegmatites 2,500 Myr ago at about the time of metamorphism^{3,4}. Sobotovich *et al.*^{5,6} reported a 4,000-Myr Pb isochron age for six whole rock samples of enderbite and granulite of the Napier complex at Fyfe Hills (67°22'S, 49°12'E). This age was calculated assuming a two-stage development of the U–Pb system, an assumption that has been invalidated by the evidence for a 2,500 Myr metamorphism⁴. Nonetheless, recalculation of Sobotovich *et al.*'s data, assuming a three-stage development, yields a minimum age of 3,500 Myr (ref. 4).

If this age is correct, the Napier complex would represent the first documented example of an Archaean granulite-facies metamorphic event that significantly post-dated crust formation. This in turn places a new perspective on the mode of formation of the early crust and the causes of granulite-facies metamorphism. In view of these implications, we have reanalysed Sobotovich *et al.*'s Fyfe Hills samples for U–Pb isotopes, and also analysed them for Rb–Sr and Sm–Nd isotopes. U–Pb isotopic systematics are often ambiguous in granulite terranes⁷. However, Sm–Nd isotopes can place more stringent constraints on the age of the complex⁸. Furthermore, the concurrent study

of the entire suite of chemically diverse elements provides a much better understanding of the geological processes which affected this part of the Antarctic crust during the Archaean. We have also analysed zircons in one of the Fyfe Hills granulites for U–Pb isotopes to document further the ~2,500-Myr age of metamorphism inferred from previous measurements on pegmatite minerals⁴.

Samples

Grikurov provided seven samples (28-I to 28-VII) collected by Kamenev and co-workers in 1970–71, and Kamenev provided a sample 28-VIII, collected in 1978. The samples include garnet-two pyroxene granulite (28-I, IV), magnesian pyroxene granulite (28-II, III), quartzofeldspathic granulite (28-VI, VII and VIII) and magnetite–garnet–orthopyroxene ironstone (28-V) (Table 1). The granulites are medium grained (0.1–3.5 mm) and lack a marked foliation while the ironstone has a cataclastic fabric. The mineral identified as perrierite or chevkinite in 28-VII forms rare brown isotropic (metamict) grains containing major Si, Ti and Ce, and subordinate Ca, Al and Th (energy dispersive scan). Minor biotite, chlorite, hornblende, or a low Ca-amphibole appear to result from post-metamorphic alteration.

Table 1 Modes* of the Fyfe Hills rocks and calculated distribution coefficients

Rock type Sample no.	Garnet-two pyroxene granulite		Magnesian pyroxene granulite		Quartzofeldspathic granulites			Ironstone 28-V
	28-I	28-IV	28-II	28-III	28-VIII	28-VI	28-VII	
Quartz	2	3	—	—	48	72	12	50
K-feldspar	—	—	—	—	—	—	15	—
Plagioclase	66	59	71	77	30	25	66	—
Orthopyroxene	5	10	28	20	9	3	5	20
Clinopyroxene	17	12	—	1	T	T	—	—
Colourless amphibole	—	—	T	T	—	—	—	—
Hornblende	—	T	—	—	—	T	T	—
Garnet	7	12	—	—	12	—	—	15
Biotite	T	T	1	2	T	T	T	T
Chlorite	—	—	—	—	T	—	T	—
Opaque	3	4	T	—	1	T	1	15
Apatite	T	T	—	—	—	—	1	T
Zircon	T	—	—	—	T	—	T	—
Monazite	—	—	—	—	—	—	T	—
Perrierite or chevkinite	—	—	—	—	—	—	T	—
D^{Rb}	0.05	0.05	0.08	0.11	0.03	0.02	0.27	<0.01
D^{Sr}	1.2	1.1	1.3	1.4	0.6	0.5	1.5	<0.1
D^{Nd}	0.185	0.120	0.024	0.029	0.115	0.032	0.279	0.109

* Visual estimates (volume %). T, trace amounts.

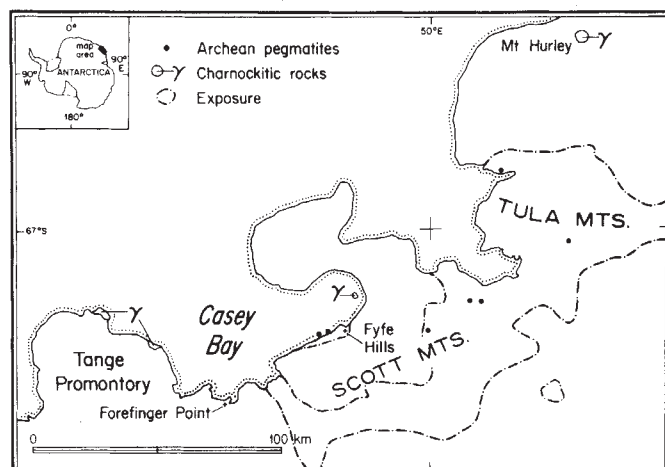


Fig. 1 Map of a portion of the Napier complex in Enderby Land showing Archean pegmatites of possible plutonic origin³¹ and intrusive charnockitic rocks (γ) on Tange Promontory (ref. 1, p. 494; ref. 3), east end of Casey Bay (J. W. Sheraton, personal communication), and Mt Hurley (ref. 1, p. 494). Specific localities of Soviet samples provided by Ye. N. Kamenev (personal communication).

The rocks vary considerably in major and trace element composition, especially in their SiO_2 content and in the relative proportions of Fe and Mg (Tables 1 and 2). Samples I and IV have igneous compositions corresponding to Fe-rich tholeiitic basalt. The other rocks, with the exception of the ironstone (sample V) have compositions that are broadly igneous except for a notable deficiency in Na_2O and K_2O . In general, the rocks are especially poor in K_2O , Rb and U, have low values of Rb/Sr and U/Pb, and high values of Th/U and K/Rb. All of these features are characteristic of granulite facies rocks^{9,10}. An additional feature is the low Sr abundances of all the rocks, some three or more times lower than typical crustal abundances¹¹. Sample VII, the only sample containing significant K-feldspar, is unique in that it contains much higher concentrations of K, Rb, Th and Nd than the other rocks, and has a high Rb/Sr ratio. Only sample VII has high U/Pb and low Th/U values. From the chemical compositions, we infer the protoliths for the samples to be volcanic rocks or immature detritus shed from an igneous terrane. The ironstone is clearly of sedimentary origin. The protolith for the magnesian pyroxene granulites is enigmatic. Their chemical compositions are not similar to igneous rock compositions, as both granulites have relatively high Mg/Fe ratios and Al_2O_3 contents, and low alkali contents.

Analytical techniques and data representation

The isotopic data were obtained using techniques described previously¹²⁻¹⁴. The U-Th-Pb and Rb-Sr data were obtained at the University of Texas; the X-ray fluorescence and Sm-Nd data were obtained at the University of California at Los Angeles. The Nd isotopic data were monitored for internal consistency by measuring all seven isotopes in all runs. Tracers for Sm and Nd concentration determinations were calibrated against gravimetric standard solutions using ultrapure metals from the Ames Laboratory^{15,16}. All Sm-Nd analyses were by the total spike method. The measured $^{143}\text{Nd}/^{144}\text{Nd}$ ratios are expressed here in the ϵ -notation:

$$\epsilon_{\text{Nd}}(0) = 10^4 \left[\frac{(^{143}\text{Nd}/^{144}\text{Nd})_{\text{sample}}}{0.511836} - 1 \right]$$

We also use the Sm-Nd enrichment factor, defined:

$$f_{\text{Sm}/\text{Nd}} = \left[\frac{(^{147}\text{Sm}/^{144}\text{Nd})_{\text{sample}}}{0.1967} - 1 \right]$$

The values in the denominators are the isotopic ratios determined for average chondritic meteorites¹⁷. Our ϵ_{Nd} values as

defined here are comparable with those of ref. 16 to within ± 0.1 units.

Results

Sample 28-VIII contained abundant rounded, unzoned zircon for which U-Pb data have been obtained (Table 3). Two fractions were dated. The 100-200 mesh fraction is nearly concordant at 2,500 Myr (Fig. 2). The >100 mesh fraction is discordant and indicated an age of 2,550 Myr on the basis of a discordia line passing through 500 Myr (by analogy with the pegmatite minerals of Grew and Manton⁴). A 2,500-Myr age for the granulite-facies metamorphism, which Grew and Manton⁴ inferred to be nearly coeval with the 2,500-Myr-old hypersthene-bearing pegmatites found throughout the Napier complex, is supported by the ages obtained from the zircons in this study. These zircons appear to have been reset at about the same time the pegmatites were emplaced. We have taken the 2,550-Myr value as the time of metamorphism for the purpose of discussion of the other isotopic data. It has been proposed that the Napier complex was also affected by a granulite-facies metamorphism about 3,000 Myr ago^{18,32}, but a second metamorphic event antedating 2,550 Myr would not affect the following arguments significantly.

Figure 3 shows the isotopic evolution of the samples as a function of time. We represented the data for each of the three isotopic systems in an analogous fashion. In all three plots, the trajectory labelled 'Mantle' represents an approximation to the isotopic evolution of the parental reservoir from which continental crust is derived by magmatic processes. It is assumed that any segment of continental crust must have had isotope ratios that lay on the mantle lines at the time it was originally derived from the mantle.

The Sm-Nd isotopic data (Fig. 3a) give the most direct indication of an ancient age of crust formation. The measured values of $\epsilon_{\text{Nd}}(0)$ (Table 2), shown at $T=0$, are extrapolated back in time using the measured values of $^{147}\text{Sm}/^{144}\text{Nd}$. The points at which these lines intersect the mantle curve correspond to the Sm-Nd model crust-formation ages (T_{DM}) (ref. 8). We have used T_{DM} rather than T_{CHUR} (ref. 19), because there is an increasing amount of evidence that continental crust is derived mainly from depleted mantle (DM), even as far back as the Archaean^{8,17,20}. The model ages clearly show that crust formation predated 3,100-Myr ago. Probably much of the crust represented by these samples formed before 3,300 Myr. The spread of model ages does not allow precise definition of crust-formation ages for these rocks. Nonetheless, the data clearly confirm the existence of early Archaean crust at Fyfe Hills. The interpretation of the T_{DM} ages is discussed further below.

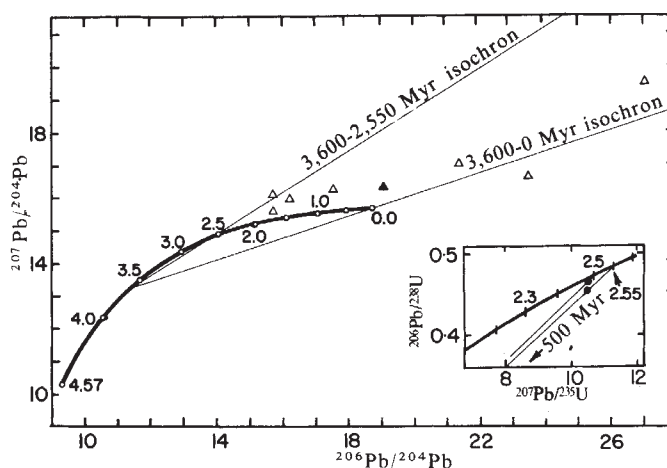


Fig. 2 $^{207}\text{Pb}/^{204}\text{Pb}$ versus $^{206}\text{Pb}/^{204}\text{Pb}$ diagram showing assumed mantle growth curve¹⁹ and data from the Enderby Land rocks. Δ , Fyfe Hills; $\bullet$, K-feldspar from pegmatite at Forefinger Point ($67^{\circ}37'\text{S}$, $48^{\circ}04'\text{E}$) (see ref. 4). Inset, portion of the concordia plot showing zircon data from sample 28-VIII.

Table 2 Chemical composition and isotope ratios of Fyfe Hills rocks

Rock type Sample no. Field no.	Garnet-two pyroxene granulite		Magnesian pyroxene granulite		Quartzofeldspathic granulite		Ironstone	
	28-I 28	28-IV 28e	28-II 28g	28-III 28d	28-VIII —	28-VI 28a	28-VII 28v	28-V —
SiO ₂ (%)	51.02	50.00	48.30	47.83	70.00	73.94	63.61	50.29
Al ₂ O ₃	14.32	13.34	19.50	22.38	12.86	13.58	14.67	3.40
CaO	10.11	9.01	8.95	12.29	5.59	3.93	4.81	0.89
Na ₂ O	2.77	2.20	1.39	1.02	1.85	2.47	2.32	ND
K ₂ O	0.42	0.40	0.23	0.40	0.38	0.38	3.02	0.15
Fe ₂ O ₃	14.00	17.80	9.90	4.43	5.70	3.51	6.41	40.78
MgO	5.64	5.43	11.34	11.25	2.35	1.48	3.25	3.24
TiO ₂	1.11	1.05	0.15	0.07	0.70	0.37	0.93	0.64
MnO	0.193	0.351	0.199	0.095	0.099	0.055	0.087	0.208
P ₂ O ₅	0.19	0.10	ND	ND	0.17	0.04	0.45	0.17
Cr (p.p.m.)	132	103	329	222	60	50	60	286
Ni	138	94	188	110	69	55	31	46
Zr	164	60	27	2	490	38	450	ND
U (p.p.m.)	0.15	0.16	0.02	0.07	2.44	0.09	3.52	0.23
Th	2.90	1.64	0.24	1.92	4.06	1.04	107	2.38
Pb	3.72	8.91	7.47	5.08	5.73	5.72	29.7	2.76
K	20.2	10.9	13.5	26.5	1.73	11.5	31.4	10.5
²⁰⁶ Pb/ ²⁰⁴ Pb*	16.17	15.68	15.73	17.57	21.32	17.50	27.05	23.46
²⁰⁷ Pb/ ²⁰⁴ Pb	15.91	15.61	16.04	16.24	17.04	16.26	19.52	16.65
²⁰⁸ Pb/ ²⁰⁴ Pb	40.60	36.09	35.50	36.92	42.46	38.20	126.6	64.91
Rb (p.p.m.)	2.63	1.09	3.66	13.4	2.25	2.03	76.2	0.31
Sr	105	124	128	44.8	110	116	103.6	6.77
⁸⁷ Sr/ ⁸⁶ Sr*	0.7120	0.7078	0.7164	0.7372	0.7134	0.7157	0.8160	0.7189
Nd (p.p.m.)	—	15.57	8.41	1.46	21.41	4.70	187.65	16.52
¹⁴⁷ Sm/ ¹⁴⁴ Nd	—	0.1566	0.0909	0.1112	0.1169	0.0782	0.0889	0.0864
ε _{Nd} (0)*	—	-19.2	-43.4	-36.6	-32.1	-46.9	-47.0	-41.9
T _{DM} (Myr)†	—	3,870	3,310	3,450	3,280	3,200	3,490	3,090

Sample numbers refer to the numbers as supplied to us. The Soviet field numbers were deduced from Table 5 of ref. 6 in which the same Rb-Sr data are presented.

All Fe as Fe₂O₃. ND, none detected. U, Th, Pb, Rb, Sr, Sm, Nd measured by isotope dilution; all other by X-ray fluorescence.

* Analytical precision is ± 0.0003 for ⁸⁷Sr/⁸⁶Sr, ± 0.5 for ε_{Nd}(0), and $\pm 0.1\%$ for Pb isotopic ratios.

† Uncertainty in model ages is typically 1–2%.

Our U-Pb and Rb-Sr data (Fig. 3b, c) also require that crust formation substantially predates the granulite-facies metamorphism. For U-Pb, the extremely low measured μ values ($\mu = {}^{238}\text{U}/{}^{204}\text{Pb}$) give calculated ²⁰⁶Pb/²⁰⁴Pb ratios that lie above the mantle evolution curve at 2,550 Myr ago²¹. Consequently, the rocks must have had much higher μ values during all or part of the time interval between crust formation and 2,550 Myr ago. The broken lines on Fig. 3b show the Pb isotopic evolution before 2,550 Myr for an arbitrarily assumed crust formation age of 3,600 Myr. The measured μ values, compared with those calculated for the time interval between crust formation and metamorphism, suggest that μ decreased by factors of 2–100 during the metamorphism as a result of severe U loss. This conclusion is independent of the value assumed for the crust-formation age. If instead of using the measured μ values, we use the measured ²⁰⁶Pb/²⁰⁴Pb and ²⁰⁷Pb/²⁰⁴Pb ratios to calculate a self-consistent set of values for μ_1 (3,600–2,550 Myr ago) and μ_2 (2,550 Myr ago to the present, see ref. 7), the calculated values of μ_2 agree with the measured values to within a factor of two. This agreement indicates that the low measured μ s are a real feature that cannot be explained by loss of U in the outcrop by recent weathering. Sample 28-VIII differs in that it apparently has recently acquired a high μ value, possibly because of a gain in U, for Pb loss is ruled out by the Th-Pb data.

The Rb-Sr data (Fig. 3c) are analogous to the U-Pb data because the calculated ⁸⁷Sr/⁸⁶Sr ratios at the time of granulite metamorphism lie well above the mantle curve. This requires the rocks to have had high values of ⁸⁷Rb/⁸⁶Sr over an extended time interval before 2,550 Myr ago. ⁸⁷Rb/⁸⁶Sr apparently decreased during the granulite facies event by factors of 10–20 in most of the rocks. The marked loss of U and Rb observed in the Napier complex is characteristic of granulite grade terranes but is more extreme than examples from younger terranes. The resultant changes in μ and ⁸⁷Rb/⁸⁶Sr cause the model ages, as determined by the point of intersection of the sample

evolution line with the mantle evolution line (Fig. 2), to be either unreasonably young (U-Pb) or undefined (Rb-Sr and some U-Pb data). In contrast, the Sm/Nd model ages are not shifted by more than $\sim 10\%$.

The U-Pb isotopic data are displayed in Fig. 2. Our analyses confirm the high ²⁰⁷Pb contents reported by Sobotovitch *et al.*⁶ but do not reproduce the linear array which was the basis of their 4,000 Myr age. The Th and Pb concentrations reported here are similar to those reported by Sobotovitch *et al.*⁶, but our U concentrations are lower by nearly an order of magnitude (except for sample 28-VII). The data (Fig. 3c) would be reasonably consistent with a model of crust formation at $\sim 3,600$ Myr ago and metamorphism at 2,500 Myr (3,600–2,500 Myr isochron), but this evidence is not sufficient to fix the crust-formation age.

Discussion

There is little evidence from other areas for metamorphic disturbance of Sm-Nd ages^{8,17,19}. Our Sm-Nd data could be interpreted, therefore, to indicate that the protoliths of the Fyfe Hills granulites represent crust formed in multiple events over a large interval of time (3,090–3,870 Myr). However, geochronological data from other areas indicate that crust formation in a given continent or province within a continent is distinctly episodic, typically lasting ~ 100 Myr (see refs 7, 8 and 22). Thus, we wish to consider the evidence that some of the scatter of T_{DM} ages observed at Fyfe Hills is a result of Sm-Nd redistribution during the granulite-facies metamorphism.

The metamorphic effects can be modelled for a rock sequence having a uniform crust-formation age, τ , for Sm-Nd and subsequently subjected to redistribution of Sm and Nd isotopes at some age $\tau_m < \tau$. We will assume that in the affected volume of rock, V, which has an overall value of the Sm/Nd enrichment

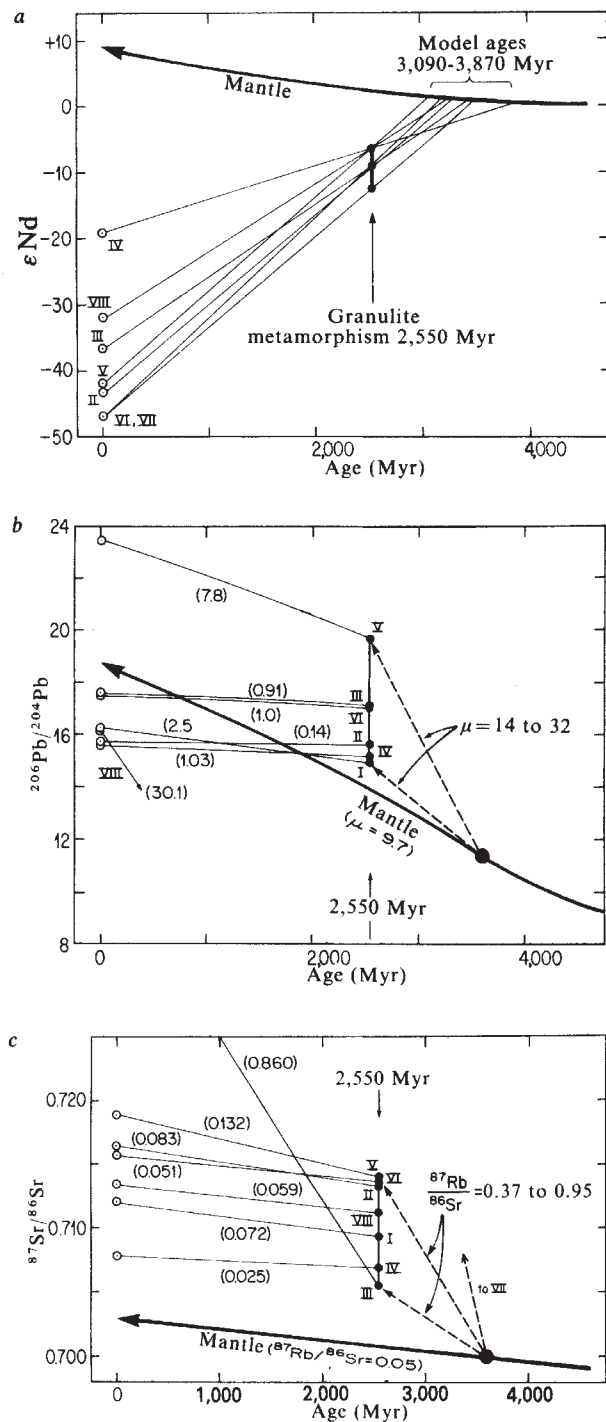


Fig. 3 *a*, Nd isotopic evolution of Fyfe Hills samples, showing model ages. *b*, Evolution of $^{206}\text{Pb}/^{204}\text{Pb}$ in Fyfe Hills samples. For illustration, the crustal evolution is shown to commence at 3,600 Myr ago (large ●). The $\mu (= ^{238}\text{U}/^{204}\text{Pb})$ values required during the time interval 3,600–2,550 Myr ago are much higher than the measured μ values, shown in parentheses. Sample VIII shows evidence for recent gain of U. *c*, Evolution of $^{87}\text{Sr}/^{86}\text{Sr}$ in Fyfe Hills samples. The range of $^{87}\text{Rb}/^{86}\text{Sr}$ ratios required during the interval 3,600–2,550 Myr are given at the right; the measured values are shown in parentheses. Large ●, crust-formation age (see *b*).

factor equal to $f_{\text{Sm}/\text{Nd}}^V$, Nd isotopic redistribution is complete, so that ϵ_{Nd} is uniform in V at time τ_m . If the volume of a particular analysed rock specimen is $\nu < V$, then $f_{\text{Sm}/\text{Nd}}^V$ may differ from $f_{\text{Sm}/\text{Nd}}^V$ by a factor, β , depending on the mineralogy

in the volume, ν . The model age, T_{DM}^V , is related to τ , the crust-formation age, by:

$$\tau \approx \tau_m + (T_{\text{DM}}^V - \tau_m)\beta \quad (1)$$

If an arbitrary τ value of 3,500 Myr and $\tau_m = 2,550$ Myr are used for the Fyfe Hills samples, β is calculated to be 0.72–1.46 for six of the samples, and 1.76 for sample V, the ironstone. With the exception of sample V, the calculated β values are not unreasonable, considering the relatively small sample size (10^{-4} m^3) and the variable mineralogy (Table 1). The β values depend on the assumed age, τ , but are plausible for any τ in the range 3,200–3,800 Myr.

The Fyfe Hills rocks show clear evidence for bulk removal of U and Rb, and thus the volume, V , for redistribution may have been large (km^3). Large-scale transport probably requires either magma or a volatile-rich fluid which can move through the rocks and selectively leach out certain elements. In this situation, which is analogous to ion-exchange chromatography²³, the average transport distance for any element will vary roughly as D^{-1} where D is the solid-fluid distribution coefficient. For a given volume of rock undergoing this metamorphic leaching, the amount of change in the elemental ratios Rb/Sr, U/Pb and Sm/Nd will depend on the absolute value of D for each element and the integrated fluid/rock ratio. For any volume of rock, ν , a bulk rock value of D_ν^α for element α could theoretically be calculated from the mineralogy:

$$D_\nu^\alpha = \sum_p D_p^\alpha \omega_p^\nu \quad (2)$$

where: ω_p^ν is the weight fraction of mineral p in volume ν , and where D_p^α is the mineral-fluid distribution coefficient for element α in mineral p . Assuming local equilibrium, if $D^\alpha \ll 1$, even a small amount of fluid would quickly shift the concentration of α , C_ν^α , to a value of:

$$C_\nu^\alpha = D_\nu^\alpha C_{\text{fluid}}^\alpha \quad (3)$$

whereas if $D^\alpha \gg 1$, C^α could be essentially unaffected by the fluid interaction.

For our hand-sized samples from a single large outcrop in the Fyfe Hills, a gauge of mobility for an element would be the extent to which $C^\alpha \propto D^\alpha$ for all the samples. In practice, this test is difficult because D_p^α values are poorly constrained, and depend on fluid composition. Nevertheless, an estimate is possible because of the relatively simple mineralogy of the Fyfe Hills samples (Table 1). D_p^α values are available for mineral/magma partitioning for Rb, Sr, Sm and Nd (refs 24, 25). The absolute values are likely to be very different for an intergranular fluid in rock undergoing granulite-facies metamorphism, but the relative values for different minerals would be similar, except for possible temperature effects ($\log D_1^\alpha/D_2^\alpha$ would be approximately proportional to inverse absolute temperature).

Computed values for D^{Rb} , D^{Sr} and D^{Nd} based on the modes are given in Table 1. For D^{Nd} , modal apatite was calculated from the measured P_2O_5 (Table 2). For Rb, where the isotopic data clearly indicate mobility, there is a significant correlation between D^{Rb} and C^{Rb} (Table 2); C^{Rb} versus modal K-feldspar + biotite also yields a good correlation. A correlation is also found between D^{Nd} (or P_2O_5) and C^{Nd} . No correlation is evident between D^{Sr} and C^{Sr} for the samples with large values of D^{Sr} , but the sample with low D^{Sr} (28-V) has low C^{Sr} , as would be predicted. These observations suggest that Nd and Sm, in addition to Rb, were mobile during the metamorphism because they were partitioned into the fluid. On the other hand, Sr was much less mobile because most of the rocks have large D^{Sr} values reflecting abundant plagioclase.

This analysis indicates that disturbance of the Sm–Nd system may have been sufficient to explain the range of observed T_{DM} ages. The Sm–Nd ages are less disturbed than those of Rb–Sr (and U–Pb) presumably because D^{Nd} and D^{Sm} are similar. In

Table 3 U–Pb Concentrations and Isotope ratios of two zircon fractions from sample 28-VIII

Fraction	U (p.p.m.)	Pb (p.p.m.)	$^{206}\text{Pb}/^{204}\text{Pb}$	$^{206}\text{Pb}/^{207}\text{Pb}$	$^{206}\text{Pb}/^{208}\text{Pb}$	$^{206}\text{Pb}^*/^{238}\text{U}$	$^{207}\text{Pb}^*/^{235}\text{U}$
> 100 mesh	630.8	326.6	1.11×10^4	5.979	6.519	0.4556	10.51
100–200 mesh	590.5	316.5	3.44×10^4	6.096	5.838	0.4662	10.55

general, the least disturbed samples should be those with large values of D for both the parent and daughter element. One of our samples (28-VII) has the requisite large values of D^{Rb} , D^{Nd} and C^{Rb} , C^{Sm} and C^{Nd} . This sample is also the only one with roughly concordant model ages for Sm–Nd (3,490 Myr) and Rb–Sr (3,740 Myr).

The T_{DM} age and the low $^{147}\text{Sm}/^{144}\text{Nd}$ ratio of sample VII provide independent evidence for some crust $\sim 3,500$ Myr old in the Fyfe Hills. An age of crust formation younger than the T_{DM} for this sample requires that $^{147}\text{Sm}/^{144}\text{Nd}$ increased during the granulite-facies metamorphism. Such an increase is unlikely given the low $^{147}\text{Sm}/^{144}\text{Nd}$ in this sample. For example, if the crust-formation age were assumed to be 100 Myr younger than T_{DM} , the protolith would have to have had $f_{\text{Sm}/\text{Nd}} = -0.63$ [equation (1)]. Rocks with such low values of $f_{\text{Sm}/\text{Nd}}$ are extremely rare; the lowest value reported is -0.61 (ref. 26) and the mean for crustal rocks is about -0.35 (ref. 11).

Conclusions

The evidence for Nd mobility during granulite-facies metamorphism demonstrates that Sm–Nd model ages cannot be taken at face value in high-grade terranes. However, because of the relatively small net shifts in the model ages, Sm–Nd still provides a much better means of determining crust-formation ages than does either Rb–Sr or U–Pb. We have too few data from the Fyfe Hills to fix precisely the crust-formation age or determine the number of crust-forming events. Based on our analysis, the best indication of the crust-formation age is probably provided by T_{DM} of 3,500 Myr for sample VII. It is not clear whether this age, if correct, would apply to all of the rocks sampled. Reported isotopic evidence for an additional event at $\sim 3,000$ Myr ago^{18,32} allows the possibility that crust formation occurred at that time also.

We conclude that the geological history of the Fyfe Hills commenced with the differentiation of magmatic rocks from the mantle at a time tentatively estimated to be $\sim 3,500$ Myr ago. The igneous crust must have become chemically layered at or about the time of formation with enrichment of Rb, U and K, and high Rb/Sr and U/Pb in the upper crust. During granulite-facies metamorphism $\sim 2,500$ Myr ago, the rocks lost most of their Rb and U, profoundly disturbing the isotopic systematics of Sr and Pb.

Our data show that a granulite-facies metamorphism post-dated crust formation by $\sim 1,000$ Myr in these rocks. Comparable time separation between crust formation and granulite-facies metamorphism has been demonstrated previously for Proterozoic granulite events^{7,27}, but not for an

Archaean ($> 2,500$ Myr) event. In the Archaean granulite terranes for which sufficient geochronological data are available^{22,28}, crust formation and granulite-facies metamorphism appear to have been contemporaneous within the resolution of the dating techniques (~ 100 Myr). Hamilton *et al.*²⁸ have attributed this to rapid heating after crust formation caused by *in situ* radioactive decay of U, Th and ^{40}K . Such a heat source is a possible cause for granulite-facies metamorphism that does not require plate tectonics. However, the conclusion was based on an erroneous heat flow calculation. Hamilton *et al.*²⁸ modelled the crust as a semi-infinite solid, whereas it should be modelled as a finite-thickness slab. In either case, *in situ* radioactive decay is not a satisfactory heat source for a granulite-facies metamorphic event post-dating crust formation by 1,000 Myr.

In general, the continental crust cannot reach granulite-facies conditions at depths corresponding to pressures of 7–10 kbar unless there is heat supplied from deeper levels. One possible heat source is magma, which in orogenic zones rises from the mantle into the crust on a regional scale over an extended period of time²⁹. Another mechanism for generating granulite-facies conditions is large-scale underthrusting in a Himalayan-type continental collision zone³⁰. Either of these mechanisms can account for a long time delay before metamorphism, and both require plate tectonics. In orogenic zones associated with subduction (magmatic arcs), granulite-facies metamorphism would be closely associated with crust formation, and at Andean-type continental margins old rocks comprising the continent may also be metamorphosed at a time much later than the time they were differentiated from the mantle. The Napier complex may represent the site of a former continental margin magmatic arc, or a continental collision zone that was active $\sim 2,500$ Myr ago, and resulted in metamorphism of $\sim 3,500$ -Myr-old continental crust. If it were a magmatic arc, we would expect to find evidence in the Napier complex for the production of new crust in the form of magma $\sim 2,500$ Myr ago. Rocks of possible plutonic origin in the Napier complex, such as intrusive charnockite (Fig. 1 and ref. 3) should be investigated. Further isotopic studies of the Napier complex and other Archaean granulite terranes presently exposed in crystalline shields could provide important evidence that a plate tectonic regime similar to the Wilson cycle was operative in Archaean time.

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An inter-laboratory comparison of radiocarbon measurements in tree rings

International Study Group*

Results from a collaborative study designed by the Glasgow ^{14}C laboratory to assess experimental variability in radiocarbon dating are presented. A series of eight replicate samples from one tree were dated independently by 20 ^{14}C laboratories. While the results are in general agreement they reveal the existence of systematic bias and unexplained variability. Thus as a general guideline for users of ^{14}C dates, quoted errors, particularly those derived solely on the basis of the counting procedure, should be multiplied by a factor of between 2 and 3.

THE method of radiocarbon dating, since its practical inception ~30 yr ago, has seen considerable development in methods of ^{14}C assay and in archaeological and geological applications requiring ever-more precise measurement¹⁻³. The corresponding increase in the number of laboratories performing such work has not, however, produced an equivalent emphasis on a necessary programme of inter-laboratory checks on analytical methods. Thus the normal procedure for assessing errors in laboratory procedures using replicate sample analysis has been long neglected in the radiocarbon dating field. This project represents the first collaborative attempt to rationalize and quantify by experiment the uncertainty involved in ^{14}C dating of a typical sample and thus its major aims have been to collect quantitative information on the variability of ^{14}C dates and to estimate the extent of inter-laboratory variability.

Organization and design of the study

Although there are at present over 100 laboratories⁴ providing a ^{14}C dating service using various analytical techniques, the logistics of the study required initial selection, at a fairly arbitrary level, of an, at least approximately, representative subgroup of laboratories, covering most, if not all, of the common experimental procedures. To this end, 30 laboratories were invited to participate in the study, with ultimately, results from 20 laboratories being submitted. The participants are listed in Table 1. It was agreed, as a prerequisite of participation, that because the aims of the study were not directed towards assessing errors in individual laboratories but rather towards monitoring group performance, anonymity would be preserved and laboratories would be assigned code letters A–T. Included in the original group of 30 was a smaller subgroup of 'high precision' laboratories, three of which agreed to take part.

The design of the study envisaged that each laboratory would date eight samples, hereafter referred to as time points 1–8, selected from a wood section spanning ~200 yr growth. Each sample, of 10 rings width, would be identified on a tree ring width plot to ensure that the same samples would be assayed by each laboratory. The sections of oak were themselves taken from one tree trunk from a submerged forest exposure at Stolford, UK (51°12' N, 3°06' W). The wood was well preserved, available in considerable quantity and sensitive ring patterns enabled accurate cross-matching of subsections. Each European laboratory received a complete section, while for reasons of postal economy non-European laboratories received the samples already extracted from their sections.

To reduce possible sources of variability in the results, each laboratory was asked to extract and date the cellulose fraction of the samples, and to this end, a standard recipe⁵ involving KOH digestion followed by $\text{NaClO}_2/\text{HCl}$ bleaching, was circulated. A few laboratories chose not to extract cellulose preferring to adopt alternative procedures. However, sub-

sequent additional work by both the La Jolla and Uppsala laboratories indicated that the method of pretreatment was, in this case, not important. Apart from pretreatment, each laboratory was asked to treat the replicate samples routinely and, in addition, to spread the measurements over as long a time period as possible to model more precisely the normal mode of operation.

The information requested from each laboratory included $\delta^{13}\text{C}$, which is the per mil deviation of the $^{13}\text{C}/^{12}\text{C}$ ratio from the PDB (belemnite) standard ratio, d^{14}C , the per mil depletion or enrichment in ^{14}C with regard to the NBS oxalic acid standard, D^{14}C which is d^{14}C normalized for isotopic fractionation, conventional radiocarbon age (using the 5,568 yr Libby half life) and its error (based solely on the counting statistics), the overall error on the result, if different, and details of the counting method. One area in which it was considered necessary to provide clear instructions to participants concerned the age and error calculations themselves. The method of age calculation was constrained to the convention recommended by Stuiver and Polach⁶. However, no similar series of instructions exists for error calculations, some laboratories commonly quoting errors based solely on theoretical estimates of the uncertainty resulting from the radioactivity measurement process alone and others including additional error terms. Thus to ensure that results would be directly comparable a method of calculating a common error term was described, the final error being arrived at through propagation of the counting uncertainties on the sample, standard and background activities alone.

Table 1 Members of the study group

Name	Laboratory
1. V. Alexeev, A. Lavrukina, I. Smirnov, Z. Milnikova, L. Sulerzhitsky	Academy of Sciences, Moscow, USSR
2. M. Baxter†, M. Scott, T. C. Aitchison	University of Glasgow, UK
3. R. Burleigh, J. Ambers, K. Matthews	British Museum, London, UK
4. J. Evin, J. Marechal, G. Marien	University of Claude Bernard, Lyon, France
5. A. Fairhall	University of Washington, Seattle, USA
6. M. Geyh	Niedersach. Land., Hanover, FRG
7. D. Harkness	Scottish Universities Research & Reactor Centre, UK
8. T. Kankainen	Geological Survey of Finland
9. W. Mook	State University, Groningen, Netherlands
10. R. Nydal, S. Gulliksen	Norwegian Institute of Technology, Norway
11. M. Stenhouse	University of Glasgow UK
12. H. Oeschger	University of Bern, Switzerland
13. I. Olsson, M. Söderman, T. Kronberg	University of Uppsala, Sweden
14. G. Pearson	Queen's University, Northern Ireland, UK
15. H. Polach	Australian National University, Australia
16. A. Rafter, H. Jansen	Institute of Nuclear Science, New Zealand
17. M. Stuiver	University of Washington, Seattle, USA
18. H. Suess, T. Linnick	University of California, USA
19. R. Switsur	University of Cambridge, UK
20. R. Williams	University of Birmingham, UK
Dendrochronology and sample distribution	
A. Heyworth	University College of Wales, Aberystwyth, UK

* See Table 1.

† To whom correspondence should be addressed at the Department of Chemistry.

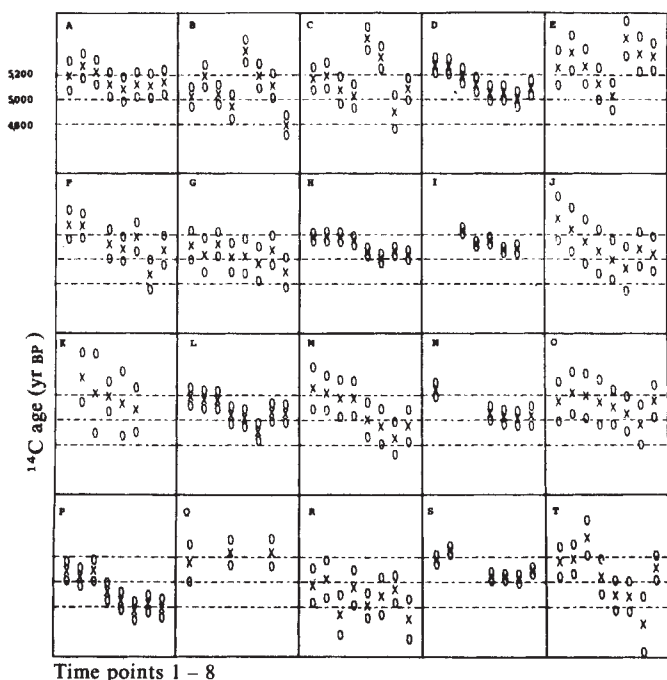


Fig. 1 Radiocarbon age (yr BP), along with 2σ error estimates versus time point for each laboratory. ---, Reference lines at 5,200, 5,000 and 4,800 yr BP.

Before discussing the results it is important to mention their limitations, these being due primarily to the small sample numbers per laboratory—eight—and to the short study period—6–9 months. Conclusions concerning individual laboratory performance can therefore only be tentative and the more general conclusions can be drawn only for this one type of material and for this specific age. In addition, only short-term variability could contribute to the observed errors. Further results, however, reported by Stenhouse and Baxter⁷ point to the relevance of the study results to routine archaeological dating over a wider range of sample types, artefact ages and laboratory time.

Results and preliminary comments

Of the results received from 20 laboratories six sets were for various reasons incomplete and thus a total of 143 measurements was obtained for this single tree. The results for each laboratory are shown in Fig. 1, the ^{14}C data across the eight time points being graphed for each laboratory, along with $\pm$ twice the quoted errors on each date. Horizontal reference lines at 5,200, 5,000 and 4,800 yr BP are shown to aid comparison. Figure 1 shows that the laboratory results all overlap in the age-range 5,000–5,200 yr BP and are thus in broad agreement. However, inspection of the results indicates the existence of considerable variability; for individual time points, results differ by between 316 and 724 yr, while, for individual laboratories, age differences of between 190 and 690 yr are observed.

The first stage of data analysis involved a preliminary estimation of the time trend through the results by calculating their weighted average at each time point, the weights in the averaging process being inversely proportional to the quoted error. The results are presented below, the trend showing the effect of ^{14}C decay during wood growth and implying the existence of a small fluctuation in mid-section.

Time point	1	2	3	4
Mean	5,168.5	5,196.1	5,173.1	5,094.4
s.e.m.	8.9	9.5	8.9	7.9

Time point	5	6	7	8
Mean	5,070.0	5,031.4	5,025.2	5,030.7
s.e.m.	7.6	7.0	8.1	8.5

Of primary interest, however, was an assessment of the variability observed about this trend relative to the claimed variability

as measured by quoted errors. This analysis was performed by estimating the 'standardized residuals'—the differences between the observed and estimated time trend divided by the quoted errors. By normalizing results in this way it is reasonable to postulate that if the quoted error does measure the true error then most, if not all, of such residuals should be within the range -2 and $+2$. Figure 2 shows the series of standardized residual plots, with horizontal reference lines at -2 , 0 and 2 . Such plots visually confirm the impressions of the existence of bias for some laboratories and of the more variable nature of others.

The relative wealth of data available, however, offers the opportunity to provide (1) a more rigorous assessment of bias and additional variability for individual laboratories (of particular interest to the ^{14}C analyst), and more importantly (2) an estimate of the variability in a typical ^{14}C date for the benefit of an archaeologist or other user.

Evaluation of results

Throughout the interpretation of the results we have chosen to model the true variability using a multiplicative factor θ , such that $\theta \times$ quoted error gives a more realistic measure of reproducibility. There are two main reasons for the choice of a multiplicative factor rather than an additive term: (1) that a multiplicative factor gives a more immediate and simple understanding of the relative magnitude of the true error involved; and (2) the estimation of such a factor θ is mathematically slightly more straightforward, particularly for interval estimates. (The intervals are a range of plausible values for θ , a property of the method being that 95% of the time they will contain the true θ value.)

Nevertheless we concede that in reality any extra variability is almost certainly additive in nature. Use of a θ factor, however, effectively produces the same results as an additive term since each laboratory has more or less constant quoted error over these samples.

The significance of θ is such that $\theta = 1$ implies that the quoted error adequately represents the true error, while $\theta < 1$ and $\theta > 1$ imply over- and under-estimation respectively by the quoted error. Point and interval estimates for θ are produced throughout by use of likelihood methods⁸.

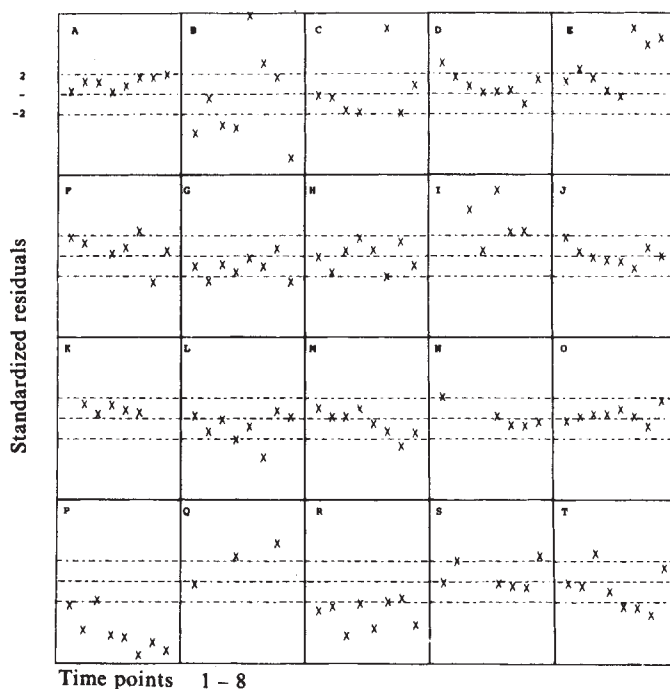


Fig. 2 Standardized residuals plotted against time point for each laboratory. ---, Reference lines at -2 , 0 and 2 .

Table 2 Point and interval estimates for bias relative to baselines (1), (2) and (3)

Lab.	Baseline (1)		(2)		(3)	
	Bias	Interval estimate	Bias	Interval estimate	Bias	Interval estimate
A	53.02	(15,90)	56.28	(35.5, 85)	52.5	(13,87)
B	-37.46	(-185,110)	-43.34	(-192,107)	-40.24	(-185,93)
C	77.38	(-90,236)	70.75	(-90,230)	74.46	(78,214)
D	24.17	(-20,65)	19.78	(-15,55)	22.63	(-12,56)
E	168.51	(36,300)	163.16	(40,302)	166.86	(36,290)
F	37.67	(-57,120)	32.85	(-60,120)	31.27	(-53,110)
G	-76.85	(-120,35)	-78.89	(-125,-35)	-77.97	(-125,-41.5)
H	-	-	-4.08	(-21,15)	-	-
I	33.62	(-10,85)	39.68	(22.5,57)	42.83	(-2,87)
J	-3.35	(-65,50)	-6.85	(-70,50)	-	-
K	72.36	(25,115)	73.71	(40,110)	72.65	(28,113)
L	-33.70	(-70,0)	-38.65	(-80,0)	-35.19	(-72,0)
M	-43.85	(-112,20)	-48.44	(-115,20)	-45.09	(-106,14)
N	6.17	(-50,60)	-4.91	(-45,30)	-	-
O	21.51	(-25,70)	14.82	(-30,55)	-	-
P	-189.63	(-240,-145)	-194.11	(-245,-150)	-191.2	(-237,-150)
Q	94.37	(-25,205)	123.19	(-10,250)	98.86	(-37,225)
R	-216.35	(-295,-145)	-219.57	(-300,-45)	-215.97	(-295,-144)
S	25.12	(-20,65)	7.40	(-25,35)	17.56	(-12,50)
T	-37.09	(-135,63)	-46.26	(-150,55)	-41.14	(-137,54)

The analysis falls into two main sections, relevant to individual laboratory and group performances. First, individual laboratories were assessed for possible bias and θ factors.

Two obvious choices of baseline and one other justifiable choice were: (1) results from high precision laboratories; (2) the weighted average of all the results, that is as before; and (3) the new weighted average of results from laboratories with identifying letters H, J, N and O giving the following results at each time point:

Weighted average	5,186	5,176	5,165	5,134
Standard error	15	19	19	18
Weighted average	5,044	4,997	5,035	5,029
Standard error	15	16	15	17

(In all cases the error on the estimates of true age were assumed to be adequately measured by the quoted errors, that is either counting errors for baseline (1) or standard errors for baselines (2) and (3).) With respect to baseline (1) only one high precision laboratory's results were used, that is H, since it had provided a complete set of eight results, while laboratory S was not quite in statistical agreement with H and I appeared systematically biased with respect to H and S). For baseline (2) no results were rejected from the study, the main reason for this being the assumption that the laboratories in the study form a representative cross-section of ^{14}C laboratories as a whole. However, laboratories showing a great deal of variability and/or bias may influence age results excessively and the baseline for the data may then best be defined by considering only laboratories whose quoted errors measure their true error and which are unbiased relative to each other. Any criterion used for rejection of results is necessarily subjective (partly due to lack of absolute knowledge about the chronology and the inadequacy of additional information concerning each laboratory's replicate error). Our approach has been to consider the two groups of laboratories which were consistent with the baselines described in (1) and (2) (the definition of consistent being that there is inconclusive evidence that bias and θ factor are different from 0 and 1 respectively). The laboratories contained in both groups were used to provide a basis for calculation of the new weighted average for baseline (3).

Results for each laboratory relative to these baselines are shown in Tables 2 and 3, the major conclusions being that: (1) Many laboratories show bias estimates ranging up to 200 yr. Only six, however, have statistically significant biases at the 5% level while three have point estimates of <20 yr. It is, of course, conceivable that, for any laboratory, bias itself may be variable with time when factors such as different standardizations and laboratory procedures are employed. The results imply that for most laboratories greater intercalibration efforts

are necessary. (2) Individual θ factor estimates range from 0.35 to 4.3, with a median value of 1.4, and thus, in general, quoted errors do not adequately express true variability. This seems to be the case even if detailed intercalibration could remove bias factors for a given laboratory. Increased emphasis on assessment of θ factors by routine replicate assays seems necessary for each laboratory.

(3) Variability in laboratories quoting errors ≥ 80 yr (that is, laboratories J, K and O) is considerably less than expected. High precision laboratories (H, I and S) are not in agreement, either through existence of a bias or through their true errors being in excess of their quoted errors. (Relative to baselines (1) and (3), θ factors for I and S are 2.6 (I), 2.0 (S) and 2.57, 1.70 respectively; relative to baseline (2), θ factors for H, I and S are 1.27, 1.08 and 1.72.)

Reasons for the differing levels of performance were investigated by considering results for each laboratory relative to baseline (1) (since results relative to other baselines do not differ significantly). The factors evaluated included type of pretreatment and method of counting. As mentioned previously, differences due to pretreatment were small. However, the method of counting did appear to be relevant, most gas laboratories having a positive bias, the liquid scintillation laboratories having a tendency to be negatively biased and most interestingly gas laboratories having smaller θ factors than liquid scintillation laboratories.

Summary statistics on the $\delta^{13}\text{C}$ measurements from each laboratory were also prepared. The average laboratory measurements ranged between -23% and -26%, with most clustered around -24%. Correlations between $\delta^{13}\text{C}$ and quoted ^{14}C ages were also calculated, several being significantly different from zero. Due to the small nature of these deviations and the both natural- and laboratory-induced sources of fractionation, no further analysis of these measurements was performed.

Analysis of the group results is, however, of greater significance to a user of ^{14}C dates because he is often required to interpret results from several laboratories and must do so with a realistic awareness of analytical errors. The subsequent analysis was therefore aimed at assessing the variability of results in small subgroups formed on the basis of level of median quoted error (m.q.e.) or method of counting. The subgroups considered were (1) m.q.e. ≤ 20 ; (2) $20 < \text{m.q.e.} \leq 45$; (3) $45 < \text{m.q.e.} \leq 75$; (4) m.q.e. ≥ 80 ; (5) gas counting laboratories; and (6) liquid scintillation laboratories (the latter two groups excluding high precision results).

The first analysis of the group results involved treating each subgroup separately, producing an estimate of bias for each individual laboratory and culminating in a common θ factor estimate for all members of the group. The results are shown

Table 3 Point and interval estimates for multiplicative factor θ relative to baselines (1), (2) and (3)

Lab.	Baseline (1)				(2)				(3)			
	$\hat{\theta}$	Interval estimate	$\hat{\theta} \times \text{m.q.e.}$	$\hat{\theta}$	Interval estimate	$\hat{\theta} \times \text{m.q.e.}$	$\hat{\theta}$	Interval estimate	$\hat{\theta} \times \text{m.q.e.}$	$\hat{\theta}$	Interval estimate	$\hat{\theta} \times \text{m.q.e.}$
A	0.84	(0.48,1.6)	48	0.57	(0.36,1.1)	29	0.83	(0.5,1.5)	42			
B	4.23	(2.8,7.7)	190.3	4.02	(2.9,7.8)	180.9	4.32	(2.6,7.2)	194.4			
C	4.10	(2.6,7.4)	233.7	3.75	(2.6,7.3)	213.75	4.09	(2.3,6.8)	233.1			
D	1.56	(0.9,2.96)	48	1.30	(0.83,2.35)	40	1.30	(0.8,2.45)	40			
E	2.64	(1.7,4.7)	184.8	2.55	(1.7,4.7)	178.5	2.63	(1.5,4.6)	184.1			
F	1.70	(1.13,3.66)	102	1.61	(1.0,3.2)	97	1.67	(1.05,3.2)	100			
G	0.81	(0.46,1.53)	48	0.94	(0.6,1.7)	56	0.91	(0.5,1.7)	54			
H	—	—	—	1.27	(0.8,2.4)	25	—	—	—			
I	2.61	(1.14,6.57)	37	1.08	(0.6,2.6)	15	2.57	(1.1,6.2)	36			
J	0.84	(0.53,1.54)	76	0.92	(0.6,1.6)	83	—	—	—			
K	0.40	(0.18,0.99)	40	0.34	(0.2,0.8)	34	0.37	(0.14,0.94)	37			
L	1.12	(0.62,2.16)	41	1.36	(0.9,2.5)	50	1.12	(0.7,2.1)	41			
M	1.15	(0.72,2.11)	80	1.17	(0.8,2.1)	82	1.07	(0.7,1.97)	75			
N	1.23	(0.53,3.3)	37	1.08	(0.6,2.4)	32	—	—	—			
O	0.77	(0.45,1.43)	62	0.68	(0.5,1.2)	54	—	—	—			
P	1.50	(1.21,2.81)	57	1.59	(1.05,2.8)	60	1.40	(0.84,2.6)	53			
Q	1.22	(0.63,3.81)	67	1.40	(0.7,4.3)	77	1.45	(0.74,4.7)	80			
R	1.37	(0.85,2.51)	96	1.51	(1.0,2.7)	105	1.43	(0.9,2.6)	100			
S	2.08	(0.88,4.88)	37	1.72	(1.0,3.5)	31	1.70	(0.9,3.8)	31			
T	2.03	(1.31,3.66)	122	2.03	(1.3,4.0)	122	1.97	(1.3,3.7)	118			

in Table 4a. Time trends through the eight samples for each group were also estimated and found, in some cases, to differ significantly. The explanation of such differences, however, is related to the presence, in some groups, of laboratories with large biases which thus affect the overall group trend.

More importantly the major implications of the Table 4a results are as follows:

(1) For the study group as a whole, the best estimate of θ is 1.85 (1.65–2.1) so that, if the laboratories in the study are representative of all ^{14}C laboratories the true error on a typical ^{14}C date may be approximately double the quoted error for a sample of $\sim 5,000$ yr age.

(2) Excluding the high precision laboratories, the value of θ decreases as quoted error (m.q.e.) increases. The value for laboratories of high m.q.e. (≥ 80) is surprisingly low, the interval estimate being completely < 1 and the true error in years being actually less than that for laboratories quoting m.q.e.s of 30–75 yr. It seems likely therefore that, for these high m.q.e. laboratories, quoted errors are being calculated on a highly conservative basis and indeed do represent overestimates. In fact, much of the importance of this inter-calibration exercise centres on the significance of quoted errors and on the lack of a universal method of error calculation. It is therefore to be desired that, some time in the future, ^{14}C laboratories should adopt a universal method of error estimation.

(3) For high precision (low m.q.e.) laboratories, while we cannot reject the possibility that the quoted error does quantify the observed variability, it is likely that, with a $\hat{\theta}$ of 1.30 and an interval estimate of 0.98–1.88, it does underestimate the true error slightly. Nevertheless, from the value for $\hat{\theta} \times \text{m.q.e.}$ of 23.4 it is evident that the procedures inherent in high precision analysis are highly significant in reducing errors; the value of 23.4 is considerably lower than the corresponding value for other groups.

(4) The estimate of θ for gas proportional laboratories is approximately half that for liquid scintillation laboratories, the implications being that, in general, the latter are more variable

and that perhaps in the future some attention might be paid to identifying the apparently uncorrected sources of variability in liquid scintillation methodology.

From (3) above, it seems likely that many of the answers may be found in the high precision procedures already adopted in the Belfast liquid scintillation laboratory.

For a user, however, information concerning relative laboratory bias is generally unknown and thus a more realistic analysis would discount possible laboratory bias and would attempt to assess the scatter of results purely in terms of θ . This is also particularly relevant since bias for any particular laboratory may well be variable through time. Using the same grouping as in the previous analysis the estimated θ values are presented in Table 4b. The values for θ have all increased as would be expected, the difference in θ estimates reflecting the effective reduction in unexplained variability when information about bias is available. The conclusions can be summarized as follows: (1) Quoted errors are inadequate for the study group as a whole, with an interval estimate for θ of 2.3–3.0, and for all subgroups other than the high m.q.e. group. The latter in fact seems to be more accurate than all but the high precision laboratories. It is clear from the range of values for θ that its value is highly dependent on the size of the quoted error and thus it is difficult to give a single 'best' value for it; use of the factor θ for the entire group supposes no information about the dating laboratory.

(2) For the high precision laboratories, a θ multiple of 1.8 is suggested and, because the interval estimate excludes 1, the quoted error represents an inadequate measure of true error.

(3) The value of θ for gas laboratories is approximately half the value for liquid scintillation laboratories so that values of 1.7 and 3.5 respectively might be recommended for users of each (for normal precision laboratories only, that is m.q.e. > 20).

Conclusions

The results from the study group provide clear indications for some laboratories at least of the existence of systematic labora-

Table 4 Group estimates of θ

Groups	<i>a</i> After bias removal				<i>b</i> No bias removal		
	No. of labs in group	$\hat{\theta}$	Interval estimate for $\hat{\theta}$	$\hat{\theta} \times \text{m.q.e.}$	θ	Interval estimate	$\theta \times \text{m.q.e.}$
1 m.q.e. ≤ 20	3	1.30	0.98–1.88	23.4	1.73	1.35–2.2	31.1
2. $20 \leq \text{m.q.e.} \leq 45$	5	2.14	1.87–2.63	81.3	3.01	2.42–3.87	114
3. $45 < \text{m.q.e.} \leq 75$	9	1.90	1.62–2.30	114	2.57	2.21–3.1	154
4. m.q.e. ≥ 80	3	0.60	0.46–0.85	54	0.71	0.54–1.01	64
5. All gas laboratories (non-high precision)	9	1.23	1.04–1.48	74	1.65	1.4–2.0	99
6. All liquid scintillation (non-high precision)	8	2.29	1.93–2.78	126	2.97	2.5–3.62	169.3
7. Entire study group	20	1.85	1.65–2.1	92	2.60	2.33–2.95	143

tory bias (both gas and liquid scintillation laboratories being equally susceptible) and of a level of variability not entirely explained by the quoted errors. One possible indicator of level of true error compared to quoted error is provided by the factor θ . The appropriate value for θ is found to be dependent on the information available; first, concerning the dating laboratory itself (knowledge of bias, counting method, size and derivation of quoted error) and, second, concerning the sample context. For the study group as a whole, the interval estimates for θ are (1) 1.65–2.1 with a point estimate of 1.85 and (2) 2.30–3.0 with a point estimator of 2.6 for situations in which (1) laboratory bias is known and has been removed and (2) no bias has been removed. In both these situations differences in performance (as measured by θ) have been observed between normal precision gas and liquid scintillation laboratories: in situation (1) above, for gas and liquid scintillation laboratories, point and interval estimates for θ are 1.23 (1.04–1.48) and 2.3 (1.93–2.78) respectively and in (2) 1.65 (1.4–2.0) and 3.0 (2.5–3.6) respectively. Thus for gas and liquid scintillation laboratories we would propose quoted error multiples of 1.2 and 2.3 for situation (1) and 1.65 and 3.0 for situation (2), the latter condition being presumably more commonly relevant.

With respect to the three high precision laboratories, there seems to be some indication that their results are more variable than claimed, but, nevertheless, their procedures do produce

significant improvements in levels of precision.

Note that this study is only a first step towards rationalization of ^{14}C dating variability. No conclusions should be overstated as each laboratory performed at most eight analyses on only one wood sample and because a great many variables remain unknown. There is, however, considerable need for further research of an intercalibrative nature involving different sample types and ages. There is apparent need also for investigation of internal replicate errors and of additional error sources (in liquid scintillation methods particularly) and for adoption of a universally acceptable method of error calculation. One should also bear in mind that in many ways the sample and laboratory procedures in this short study were close to ideal, with well preserved and sufficient sample assayed over a short period of laboratory time. Most laboratories extracted cellulose before assay, thereby presumably removing any minor cross-contamination effects. It might seem therefore that the error estimates discussed here represent minimum values which would possibly require enlargement for: degraded/small/contaminated materials; different sample types; and replicate samples counted over a longer time period. The results urge increased caution in attempting to resolve ^{14}C differences of <200 yr.

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***De novo* methylation and expression of retroviral genomes during mouse embryogenesis**

Detlev Jähner, Heidi Stuhlmann, Colin L. Stewart, Klaus Harbers, Jürgen Löhler, Iva Simon & Rudolf Jaenisch

Heinrich-Pette-Institut für Experimentelle Virologie und Immunologie an der Universität Hamburg, Martinistrasse 52, 2000 Hamburg 20, FRG

Retrovirus genomes introduced into mouse zygotes by microinjection of cloned DNA, or into morula stage pre-implantation mouse embryos by infection with Moloney murine leukaemia virus (M-MuLV), became de novo methylated and were blocked in expression. No restriction of virus expression and no de novo methylation were observed when post-implantation mouse embryos were infected with virus. Efficient de novo methylation activity may be an important characteristic of gene regulation in early mouse embryos.

THE introduction of foreign cellular and retroviral genomes into early mouse embryos has been used to investigate the regulation of gene expression in mammalian development^{1–10}. Early mouse embryos and embryonal carcinoma cells have been shown to be non-permissive for DNA virus as well as retrovirus gene expression^{11–16}. Recently, we correlated the lack of expression of genetically transmitted Moloney murine leukaemia (M-MuLV) proviral genomes with their high degree of methylation^{17,18}. As the retroviral genomes introduced into the germ line of these mice were unmethylated, *de novo* methylation of the proviral genomes must have occurred at some point, either during the development of the infected embryo and/or as a consequence of their transmission through the germ line. To investigate this, we have introduced M-MuLV genomes into pre- and post-implantation embryos. Here we show that efficient *de novo* methylation occurred after the introduction of virus into pre-implantation but not into post-implantation embryos. The resulting *de novo* methylated genomes were not

active in a DNA transfection assay and were apparently not expressed in early embryos.

Integration of M-MuLV genomes into mouse embryo

M-MuLV genomes were introduced into mouse embryos at different stages of embryogenesis (see Table 1). Pre-implantation embryos were infected at the zygote stage by injecting the cloned M-MuLV-containing *EcoRI* fragment from Mov-3 mice either into the male pronucleus (animal 69) or into the cytoplasm (animal 158)⁶ and, at the 4–16-cell stage, by exposure to M-MuLV virus⁴. Post-implantation embryos were injected at day 8 of gestation with purified M-MuLV virus as described previously¹⁹.

The number of M-MuLV genomes present in mouse DNA extracted from 2–4-month-old animals was determined by digestion with *SstI* (see Fig. 1). The intensities of the M-MuLV-

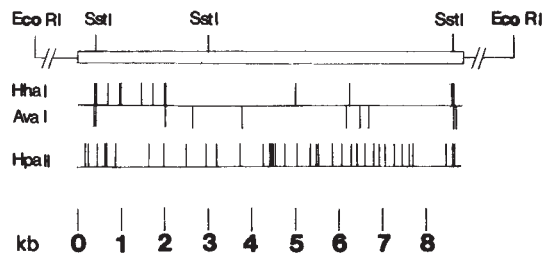


Fig. 1 Restriction enzyme map of a M-MuLV provirus. The open bar represents a M-MuLV provirus within flanking mouse DNA. Restriction sites of M-MuLV were taken from published maps⁴⁴ and sequences⁴⁵.

specific *SstI* fragments of 5.6 and 2.6 kilobases (kb) from DNA of the experimental mice were compared with those from DNA of Mov-1 mice, thus permitting estimation of the total number of M-MuLV copies present per cell, as described elsewhere⁶. Figure 2 shows the presence of approximately 10 M-MuLV copies per cell in the DNA from mouse 69 and one copy in the DNA from mouse 158. Animals infected at day 3 of development carried between one and three copies and animals infected at day 8 of development carried between one and eight M-MuLV copies per cell in different tissues (Fig. 2, Table 1; compare ref. 19).

M-MuLV proviruses become integrated at multiple sites into pre-implantation embryos^{4,5}, mouse lymphocytes²⁰ and fibroblasts²¹. Because *EcoRI* does not cut within the M-MuLV genome, different integration sites of individual proviruses are characterized by different sizes of M-MuLV-containing *EcoRI* fragments. The number and intensities of such *EcoRI* fragments were used here to determine the time at which provirus integration took place in embryonal development. A single proviral copy inserted into the genome of the zygote will subsequently be present in every cell in all tissues of the mouse, resulting in an *EcoRI* fragment with the same intensity as the fragment which represents the single-copy M-MuLV genome in Mov mice. This has, indeed, been demonstrated for mouse 158 (Fig. 3, lane b, and ref. 6). DNA from mouse 69, which was derived from a zygote microinjected with cloned Mov-3 DNA into the

male pronucleus, contained a strong band of 17 kb representing approximately 10 M-MuLV copies and a single copy fragment of higher molecular weight (Fig. 3, lane a). Further restriction enzyme analysis indicated tandem head-to-tail integration of the injected Mov-3 DNA, the single copy band representing Mov-3 DNA together with flanking mouse DNA (data not shown).

When exposure to virus takes place at a later stage (the 4–16-cell stage), integration can occur at different chromosomal sites as well as in different blastomeres. This will lead to less intense *EcoRI* fragments, depending on the segregation of the descendants of the infected blastomere during later development and their contribution to a given organ. Several *EcoRI* fragments of varying intensity were, indeed, observed in the DNA of such animals (Fig. 3, lanes c, g), indicating that these animals were mosaics with respect to the integrated M-MuLV copies (compare ref. 4). Germ-line mosaicism of such mice had been demonstrated previously^{3–5}. The results shown in Fig. 3 revealed that the M-MuLV genomes present in the DNA of mice that were infected as pre-implantation embryos integrated in only a few chromosomal sites in a given animal. This indicated that the integration of M-MuLV genomes in these embryos occurred soon after the time of infection. Little or no reintegration of viral genomes had occurred during later stages of development (see below).

A different pattern of M-MuLV-specific *EcoRI* fragments was observed after infection with M-MuLV at a later stage, for example, day 8 of development, when the embryo consists of 10^4 – 10^5 cells. A broad smear of hybridization was seen and no discrete *EcoRI* fragments were detectable in any organ of such animals (Fig. 3, lanes i, k). This suggested that integration of virus took place at multiple sites in a heterogeneous population of cells. Each infected cell divided to form only a small fraction of the total number of cells in the respective tissue. We have shown previously that after postnatal infection of mice with M-MuLV, significant amounts of proviruses were not found in cells other than those of lymphopoietic origin¹⁹. The copies of M-MuLV in the non-lymphatic organs liver, kidney and brain of day-8 infected mice (Fig. 2b) therefore indicated prenatal virus spread. This conclusion was supported by *in situ* hybridization experiments (Fig. 4). At 4 days after injection of virus, multiple foci of cells expressing viral RNA were detected in liver and brain cells as well as in cells of other tissues. Viral proteins were detected similarly in multiple cells by immunofluorescence (not shown). Thus, efficient virus replication and infection took place at mid-gestation. These results, summarized in Table 1, suggested that the M-MuLV DNA detected in the adult integrated into the mouse genome at or soon after the time of the introduction of viral genomes.

Table 1 Organization of M-MuLV copies introduced into mice at different stages of embryogenesis

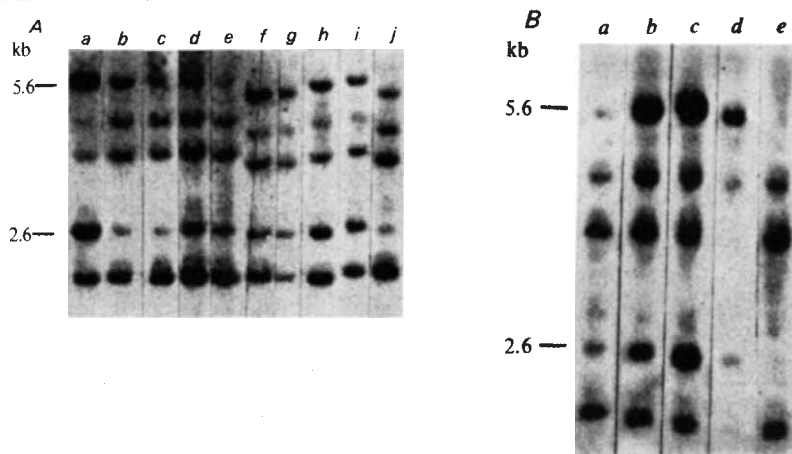
Time of infection	Animal	Organ	No. of copies	No. of integration sites
Pre-implantation stage				
Day 1	69	Li	10–15	1
	158	Li	1	1
Day 3	58–1, 58–2	Li, Ki, Br	1–3	1–3
	61, 62			
	100–19, 100–21			
Post-implantation stage				
Day 8	B58, B59	Li	0.5–1	Multiple
		Ki	2–4	
		Br	4–8	
Control	Mov-13	Li	1	1

M-MuLV genomes were introduced into pre-implantation mouse embryos by DNA injection at day 1 or virus infection at day 3 of gestation. Microinjection of the cloned 16.8-kb *EcoRI* fragment from Mov-3 mice into the male pronucleus (animal 69) or into the cytoplasm (animal 158) of mouse zygotes at the day of fertilization was performed as described for mouse 158 (ref. 6). Four- to eight-cell embryos (that is, day 3 embryos) were infected *in vitro* as described for animal 62 (ref. 4). Post-implantation embryos were injected with virus *in utero* at day 8 of gestation as described elsewhere¹⁹. The data from mice B58 and B59 were representative of several similarly infected mice. The number of M-MuLV copies per cell was obtained by *SstI* digestion with DNAs from liver (Li), kidney (Ki) or brain (Br) by comparison with the Mov-13 DNA (see Fig. 2). This DNA contained one M-MuLV genome per diploid genome. *EcoRI* digestion of DNA was used to determine the number of integration sites of M-MuLV in a given organ (see Fig. 3). All embryos were of strain C57BL/6 except animals 62, 100–19 and 100–21, which were ICR and 129 mice, respectively.

De novo methylation of M-MuLV proviruses

The methylation of CpG dinucleotides in M-MuLV genomes present in the DNA of animals infected during different stages of embryogenesis (see Table 1) was analysed with methylation-sensitive enzymes^{22–24}. Because newly synthesized retroviral DNA, the precursor of the provirus, is not methylated, the presence of 5-methylcytosine will reveal that *de novo* methylation has occurred between the time of integration and the time of DNA preparation. A given site can either be methylated or unmethylated. However, when studying the DNA from a population of cells, the same restriction site can be methylated in some molecules and unmethylated in the rest. Due to this heterogeneity, intermediate levels of methylation have been found in several analyses^{25–28}. The presence of methyl groups in M-MuLV genomes was analysed by the resistance of the M-MuLV-specific *SstI* or *EcoRI* fragments to digestion with the methylation-sensitive enzymes *HhaI*, *AvaI* or *HpaII* (see Fig. 1). In all the DNAs from animals infected as pre-implantation embryos, the 2.6-kb *SstI* fragment was highly resistant to digestion with all three enzymes (see Fig. 5). The 5.6-kb *SstI*

Fig. 2 Estimation of M-MuLV copies in DNAs from mice infected during embryogenesis. High molecular weight DNA was prepared, samples of 5–10 µg were cleaved with *Sst*I, separated on a 0.7% agarose gel and transferred to a nitrocellulose filter as described previously¹⁷. The filters were hybridized with a cDNA enriched for sequences specific for M-MuLV and washed after hybridization at 50 °C as described elsewhere²⁰. **A**, DNAs from animals infected as pre-implantation embryos. Lanes *a, b*, liver DNA from animals 69 and 158, respectively; lanes *c–e*, DNA from liver, kidney and brain, respectively, of animal 58-1; lanes *f, g*, DNA from liver and brain, respectively, of animal 58-2; lanes *h, i*, liver DNA from animals 100-19 and 100-21, respectively; lane *j*, embryo DNA from Mov-13 mice⁵, containing one copy of M-MuLV per cell. **B**, DNAs derived after infection of post-implantation embryos. Lanes *a–c*, DNAs from liver, kidney and brain, respectively, of animal B58; lane *d*, embryo DNA from Mov-1 mice, containing two copies of M-MuLV per cell; lane *e*, DNA from C57BL/6 mice. The bands present in all DNAs are due to cross-hybridization of the probe to DNA of endogenous retroviral genomes of the mouse²⁰. These bands served as an internal standard for the amount of DNA present in each lane. The intensities of the 5.6- and 2.6-kb M-MuLV-specific *Sst*I fragments in DNAs from Mov mice, **A**, lane *j* and **B**, lane *d*, were used to estimate the number of M-MuLV copies in the other DNAs (see Table 1). As Mov-1 is of BALB/c background, bands derived from endogenous viruses are weaker with this probe than in C57BL/6 (compare lane *d* with lane *j*, **A**).



fragment was similarly resistant to cleavage with *Hha*I and *Ava*I and to some extent with *Hpa*II.

From the results shown in Fig. 5A we calculated that 70–95% of each of the sites had become *de novo* methylated in these DNAs (see Table 2). Our calculation was based on the assumption that every site was methylated to the same extent. This is supported by the following observations: (1) The resistance of both *Sst*I fragments did correspond to the number of cleavage sites present. Such a cumulative effect of multiple sites argued against the presence of certain sites with low levels of methylation.

Table 2 *De novo* methylation of M-MuLV genomes at *Hha*I, *Ava*I and *Hpa*II sites in organs of animals infected during embryogenesis

Time of infection	DNA	Level of methylation at individual sites (%)	No. of sites
Pre-implantation stage	69 Li	70–95	44
	158 Li	70–90	44
	58-1 Li	70–95	22
	58-1 Ki	70–95	22
	58-1 Br	70–95	22
	58-2 Li	80–95	44
	58-2 Ki	70–90	22
	58-2 Br	70–90	22
	61 Li	70–95	44
	62 Li	70–95	22
	100-19 Li	70–95	44
	100-21 Li	70–95	44
Post-implantation stage	B58 {Li	<20	11
	B59 {Ki	<20	11
	Br	<20	11

See Table 1 legend for details of infection and organ description. The level of *de novo* methylation in M-MuLV genomes present after infection of pre-implantation embryos was determined by the resistance of the M-MuLV-specific 5.6- and 2.6-kb *Sst*I fragments to digestion with *Hha*I, *Ava*I or *Hpa*II (see Fig. 5). Only sites sufficiently distant from the *Sst*I sites to reduce the size of the *Sst*I fragments are included in the results. The 5.6-kb fragment has 25 *Hpa*II sites, the 2.6-kb fragment 7 *Hpa*II and 7 *Hha*I sites (see further in Fig. 1). With the exception of some *Ava*I sites in liver DNA of animal 69 (Fig. 5A, lane 1c), no preferential sensitivity of any site was seen. Therefore, assuming similar levels *L* of methylation in all *n* sites of a given enzyme present in an *Sst*I fragment, $L = n\sqrt{I}$, where *I* is the resistant fraction of the fragment obtained from the autoradiogram by visual inspection. If a fragment containing two sites for, for example, *Hha*I, is 70% resistant to cleavage with *Hha*I, then the level of methylation in both sites is 84%. The range in the percentage of methylation is due to differences observed with several enzymes and to the inaccuracy of determination of intensities. The level of *de novo* methylation in DNAs from animals infected at the post-implantation stage was estimated from the intensity of fragments appearing after digestion with *Hha*I or *Ava*I. The intensity of each fragment appearing is dependent on the presence of two unmethylated sites, thus $L = 1 - \sqrt{I}$. *I* was >70% for all the M-MuLV fragments, compared with fragments of similar size derived by methylation-insensitive enzymes from the same DNA (see Fig. 6). In some cases no results for the *Hpa*II digestion of the 5.6-kb fragment are given, because a low number of M-MuLV copies per cell does not allow determination due to the multiple sites (see above). Four *Hha*I and seven *Ava*I sites resulting in fragments >0.6 kb were included in the analysis of appearing fragments (see Figs 1, 6).

(2) No digestion products were found in the digests with the methylation-sensitive enzymes. Therefore, no detectable fraction of the total M-MuLV genomes had remained completely unmethylated, nor was there evidence for a number of sites methylated to a low degree. The latter conclusion was valid only for the *Hha*I and *Ava*I digestions, as *Hpa*II digestions could result in many undetectable digestion products (see Fig. 1). The above results suggested that the observed digestion of *Sst*I fragments of M-MuLV with *Hpa*II was only due to the presence of multiple sites, all of them methylated at a high level (see Table 2 for calculation) rather than to a lower level of methylation at such sites in a fraction of genomes. *Eco*RI/*Hha*I digestions of these DNAs revealed that some of the *Eco*RI fragments containing a M-MuLV provirus and several kilobases of flanking mouse DNA were highly methylated in all *Hha*I sites present (see Fig. 3, lanes *d, f, h*).

In contrast to the above results, the M-MuLV genomes that integrated in post-implantation embryos showed a low degree of *de novo* methylation. The *Sst*I and *Eco*I fragments (see Fig. 5B and Fig. 3, lanes *j, l*) were almost completely digested with methylation-sensitive enzymes. The levels of methylation in these DNAs were determined by digestions with *Hha*I or *Ava*I (see Table 2 for calculation). The intensities of the fragments appearing were compared with those of fragments of similar length derived from digestion of the same DNAs with the methylation-insensitive enzyme *Sst*I (see Fig. 6). Methylation of some of the sites would reveal fragments

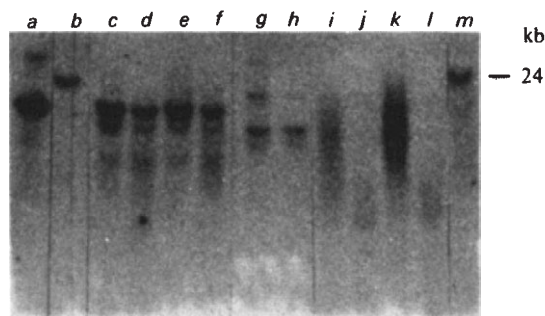


Fig. 3 Integration sites of M-MuLV genomes introduced into mouse embryos. The DNA samples were digested with *Eco*RI (lanes *a, b, c, e, g, i, k, m*) or *Eco*RI plus *Hha*I (lanes *d, f, h, j, l*). The completeness of the digestions was tested by addition of cloned M-MuLV DNA to a part of each digestion mix. The DNAs were separated on 0.5% agarose gels, a M-MuLV-specific cDNA being used for hybridization. Lane *a*, 5 µg of liver DNA from mouse 69; lane *b*, 10 µg of liver DNA from animal 158; lanes *c–f*, 20 µg DNA from animal 58-2, from kidney (*c, d*) and brain (*e, f*). Lanes *g, h*, 10 µg of DNA from kidney of mouse 100-21; lanes *i–l*, 10 µg DNA from animal B58, from kidney (*i, j*) and brain (*k, l*); lane *m*, 10 µg of embryo DNA from Mov-13 mice, containing one copy of M-MuLV per cell.

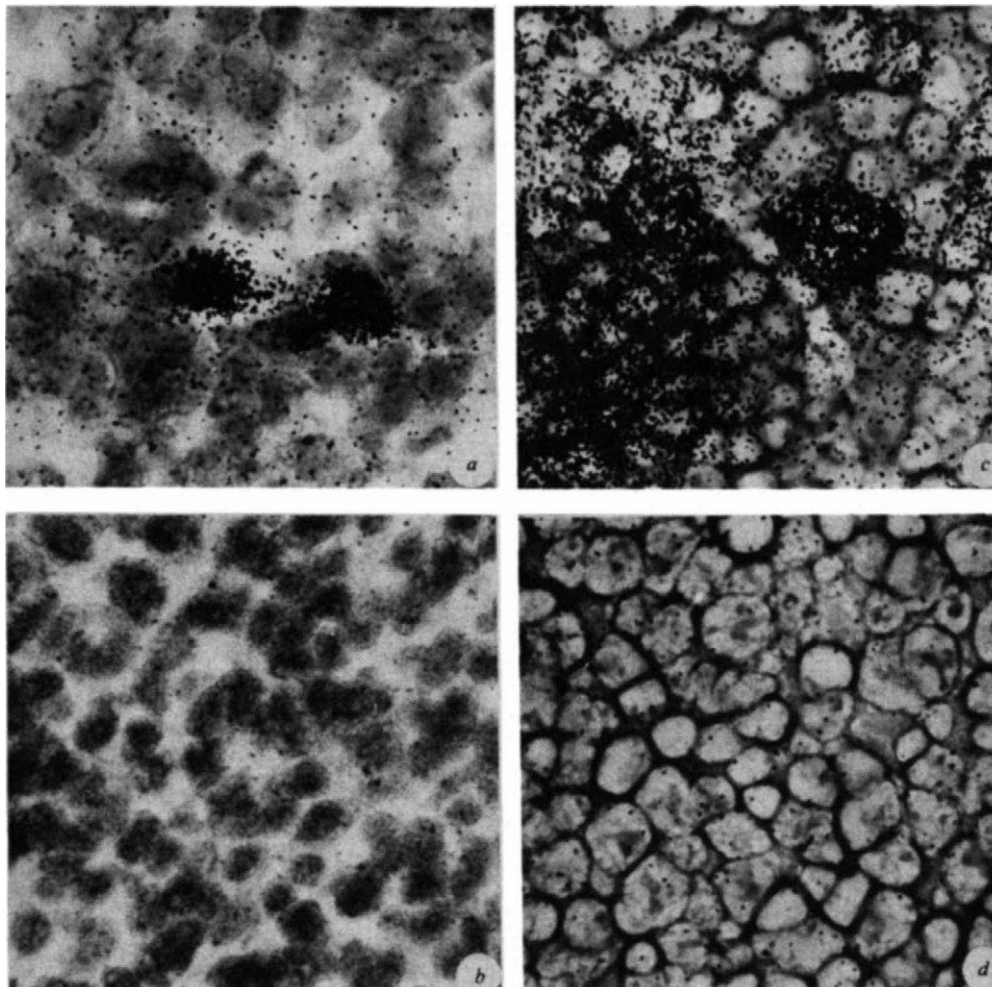


Fig. 4 Detection of M-MuLV RNA in post-implantation mouse embryos by *in situ* hybridization. Mouse embryos were infected *in utero* at day 8.5 of gestation with M-MuLV (see Table 1). Four to five days after infection, frozen sections of whole embryos were prepared, hybridized *in situ* with ^3H -labelled M-MuLV cDNA, and finally counter-stained with Giemsa stain as described previously⁴⁶. *a*, Mantle layer of cerebral cortex showing focal expression of M-MuLV RNA; *b*, cerebral cortex of control embryo; *c*, foci of viral RNA in liver tissue; *d*, liver of control embryo. $\times 486$.

differing in length and intensity from those derived from a completely unmethylated (that is, cloned) genome. Our analysis showed that at least 80% of the genomes introduced into post-implantation embryos remained completely unmethylated in 11 sites analysed. Because no partial digestion products or completely methylated genomes were visible (see also Fig. 5), we conclude that all 11 sites were methylated to less than 20% (see Table 2). Thus, these experiments demonstrated a striking

difference in *de novo* methylation of M-MuLV genomes: a highly efficient *de novo* methylating activity is present only in pre-implantation embryos but not in post-implantation embryos or after birth. Furthermore, the methylation of M-MuLV genomes introduced into pre-implantation embryos was maintained over many cell generations.

Expression of M-MuLV in infected embryos correlates with the infectivity of proviral DNA in the adult

Previous results have shown that pre-implantation mouse embryos are not permissive for virus replication^{2,6,16}. The *EcoRI* pattern of M-MuLV-specific fragments indicated that infection with M-MuLV and integration of viral DNA did occur; however, virus spread, that is, virus expression, was not detectable in early mouse embryos, but did occur later in development in an unknown population of cells, leading to viraemia in the adult (compare ref. 5). In contrast, virus integration into cells of mid-gestation embryos led to expression and efficient spread throughout cells of all tissues of the infected embryo (Fig. 4). Therefore, those viral copies found in adult animals derived from infections of post-implantation embryos have been actively expressed after integration in most of the cells.

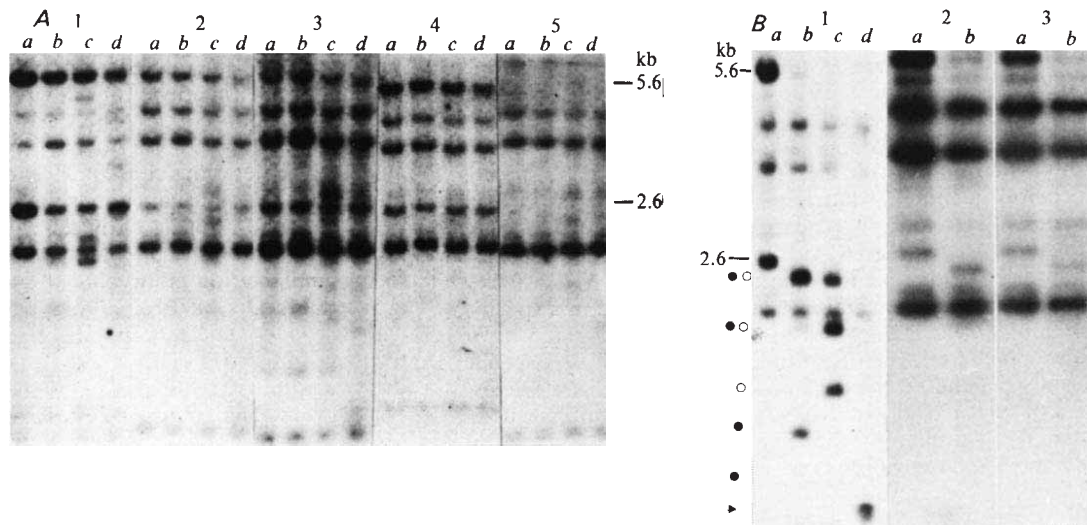
DNA transfection was used to study the biological activity of the same M-MuLV genomes as were analysed in previous sections. Table 3 shows that DNA containing M-MuLV genomes integrated in pre-implantation embryos was not infectious. The DNA from animal 69 carried 10–15 copies of pMov-3 DNA per cell. Thus, the injected DNA was several hundredfold less infectious after passage in the mouse than before injection. In contrast, the copies that were introduced

Table 3 Infectivity of DNA from organs of mice infected with M-MuLV during embryogenesis

Time of infection	DNA	No. of XC PFU	Specific infectivity $\times 10^6$
Pre-implantation stage	69 Li	0	<0.01
	58-1 Li	0	
	58-1 Ki	0	
	61 Li	0	
	100-19 Li	0	
Post-implantation stage	B58 Li	8	1.4
	B58 Ki	24	0.7
	B58 Br	46	1.0
Controls	Mov-1 Sp	10–20	1.8
	C57BL Li	0	0
	1 ng pMov-3	500	8

The number of XC plaque-forming units (PFU) is given as a mean value from 30 μg DNA tested in two independent transfection assays as previously described¹⁷. Specific infectivity of DNA, defined as the number of XC PFU per viral genome, was calculated from the number of M-MuLV copies present in the respective DNA (compare Table 1). Infectivity of DNA from preleukaemic Mov-1 spleen is due to one to two M-MuLV copies acquired postnatally¹⁷. DNA from the highly infectious pMov-3 clone (specific infectivity 8×10^{-6} PFU per viral genome)¹⁸, which was used for injection into zygotes (animal 69), was included as a control.

Fig. 5 Methylation of M-MuLV genomes. DNAs were analysed from mice infected as pre-implantation (A) or as post-implantation (B) embryos. All samples were digested with *Sst*I and also with: b, *Hha*I; c, *Ava*I; d, *Hpa*II. A, DNA from liver of mouse 69 (1a-d), liver of mouse 158 (2a-d), kidney of mouse 58-1 (3a-d), liver of mouse 58-2 (4a-d), C57BL/6 mice (5a-d). B, DNA from kidney of mouse B58 (1a-d), kidney of mouse Bv11 (2a, b), brain of mouse Bv12 (3a, b). The positions of DNA fragments of M-MuLV derived after double digestion with: ●, *Hha*I; ○, *Ava*I; ►, *Hpa*II are indicated. The cDNA was not representative of all sequences of M-MuLV; therefore, the intensities of the fragments did not correspond to their sizes (compare ref. 4).



into post-implantation embryos were as infectious as those integrated after birth. Previous results from this laboratory have correlated DNA methylation and expression of M-MuLV genomes with infectivity in a transfection assay^{17,18,29}. The results described in Table 3 indicate that viral genomes which were *de novo* methylated after integration into pre-implantation embryos are non-infectious in the adult animal. Thus, both persistence of viral genome methylation and non-infectivity may be sustained by a maintenance methylase activity^{24,30,31} for as long as 5 months after birth. In contrast, viral genomes inserted in cells of mid-gestation embryos are expressed, remain unmethylated and are infectious in a transfection assay when isolated from the adult.

Discussion

We have shown previously that pre-implantation mouse embryos are not permissive for replication of M-MuLV^{2,6}. Viral DNA, however, can integrate efficiently into the embryo DNA and mouse substrains designated as Mov-1 to Mov-13 that carry M-MuLV in their germ line have been derived from infected embryos³⁻⁵. Virus is activated in some of these substrains and the available evidence suggested that the developmental stage at which virus activation occurs depends on the chromosomal position of the provirus⁵. Further analysis showed that the genetically transmitted proviral genomes in several organs of Mov substrains were highly methylated and non-infectious in a transfection assay^{17,18}.

We have shown here that retroviral DNAs become *de novo* methylated on integration into the genome of pre-implantation embryos as assayed in organs of the adult derived from the infected embryo. This was observed after exposure of the embryos to infectious virus as well as after microinjection of cloned viral DNA with flanking mouse sequences. In contrast, virus introduced into post-implantation embryos replicated efficiently in most or all embryonal cells and the integrated proviral genomes remained largely unmethylated when assayed in the adult animal. The difference in *de novo* methylation was also reflected in the transfection assay: DNA from animals exposed to virus at the pre-implantation stage was not infectious, in contrast to DNA from animals exposed to virus at mid-gestation. Thus, our results suggest that an efficient *de novo* methylation activity may be characteristic of cells of the pre-implantation stage and may be absent or less effective in cells of later embryos. This activity correlates with non-permissivity to virus expression. Furthermore, our results show that the maintenance methylation activity is faithful in preserving the respective methylation pattern of the proviral genomes throughout the life of the animal. The *de novo* methylation activity in embryonal cells may be of general significance, as

not only viral but also globin DNA (F. Costantini and E. Lacy, personal communication) and a metallothionein-thymidine kinase fusion plasmid³² became *de novo* methylated after microinjection into mouse zygotes.

Recent experiments obtained in tissue culture systems have demonstrated that embryonal carcinoma cells have an efficient *de novo* methylation activity, in contrast to differentiated cells³³, and that methylation correlated with gene expression. In these experiments we analysed the fate of the infecting DNA: the viral DNA remained unmethylated as long as it was unintegrated and became *de novo* methylated soon after genomic integration. This is relevant for the observation that DNA microinjected into mouse zygotes^{6,34} or into *Xenopus* eggs³⁵ is

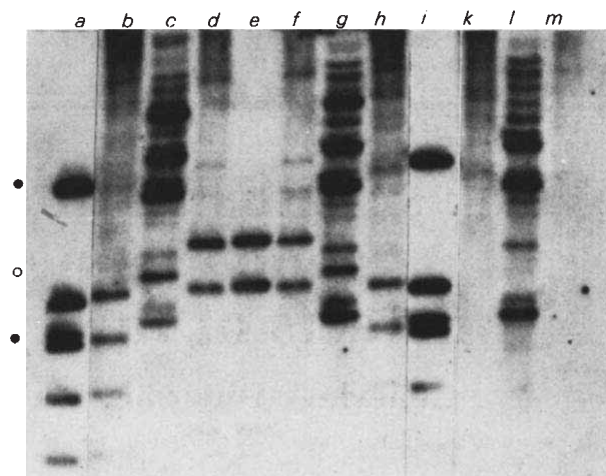


Fig. 6 M-MuLV genomes are not methylated in DNA from mice infected at the post-implantation stage. Lack of methylation was demonstrated by (1) appearance of equimolar M-MuLV-specific fragments after digestion of DNAs from mouse B59 and cloned M-MuLV DNA with *Ava*I and *Hha*I and (2) by comparing the intensity of the 2.6-kb fragment derived from digestion of DNAs from B59 with the methylation-insensitive enzyme *Sst*I to the intensity of fragments of similar size derived from digestion of the same DNAs with the methylation-sensitive enzymes *Hha*I and *Ava*I. DNA (10 µg) from brain and kidney of animal B59 were separated on a 0.8% agarose gel. The hybridization was performed with a nick-translated M-MuLV DNA. Lane a, *Ava*I-digest of plasmid p9-2, containing M-MuLV DNA²⁹; lanes b-d, *Ava*I-, *Sst*I- and *Hha*I-digested brain DNA, respectively; lane e, *Hha*I digest of 9-2; lanes f-h, *Hha*I-, *Sst*I- and *Ava*I-digested kidney DNA, respectively; lane i, *Ava*I digest of p9-2; lanes k-m: *Ava*I-, *Sst*I- and *Hha*I-digested C57BL/6 DNA, respectively. The positions of the M-MuLV-specific 2.6-kb *Sst*I fragment (○) and of the two plasmid sequences containing *Ava*I fragments of p9-2 (●), which are therefore not found in DNA from B59, are indicated.

expressed as long as it remains in an episomal state. Unintegrated DNA injected into *Xenopus* eggs was shown to remain unmethylated³⁶. So far, *de novo* methylation activity has only been observed as a rare event in differentiated cell lines after long-term cultivation to select cell clones³⁷⁻⁴⁰.

If the *de novo* methylation activity in embryonal and efficient maintenance methylation in later cells are involved in repression of proviral genomes, what is the origin of infectious virus in mice derived from pre-implantation embryos exposed to virus? Because virus, once activated, will infect all susceptible cells and spread throughout the animal, demethylation and activation of virus at a given stage of development and in a specific, as yet unidentified, population of cells would be sufficient to lead to viraemia. Demethylation of a given provirus in specific cells may depend on the chromosomal position where integration took place, and proviral genome activation may thus be regulated by similar mechanisms to those proposed for the tissue-specific activation of developmentally regulated

genes^{41,42}. Gene activation of the proviral genome in Mov-1 mice seems to be compatible with such a hypothesis⁴³.

Our results suggest that embryonal cells may possess an efficient *de novo* methylation activity that inactivates any DNA which is introduced into the early embryo. This may have evolved as a mechanism to protect the developing embryo against the deleterious consequences of virus infections. Finally, our results pose intriguing questions concerning the control of gene expression during early development, and it will be of great interest to study the methylation of genes that are active in pre-implantation embryos and embryonal carcinoma cells.

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Single base substitution in an intron of oxidase gene compensates splicing defects of the cytochrome *b* gene

Geneviève Dujardin, Claude Jacq & Piotr P. Slonimski

Centre de Génétique Moléculaire, Laboratoire propre du CNRS, associé à l'Université Pierre et Marie Curie, Paris VI, F-91190 Gif-sur-Yvette, France

An extragenic suppressor mutation, mim2-1, which compensates yeast mitochondrial mutants deficient in splicing of the cytochrome b gene, has been mapped and sequenced. The mutation is due to a single G → A transition in the long open reading frame of the fourth intron of the oxidase subunit one gene. It causes the replacement of a glutamic codon by a lysine codon and the expression of a novel mRNA maturase active in splicing. Evolution and regulatory connections between homologous introns of nonhomologous genes are discussed.

THE mitochondrial gene *cob-box* of *Saccharomyces cerevisiae*, which specifies the apocytochrome *b*, exhibits a mosaic organization of exons and introns¹⁻⁶. Respiration-deficient mutants have been mapped in both the exons and introns of this gene. Exon mutants belong to a single complementation group while intron mutants belong to separate complementation groups⁷.

Intron mutations have been shown to block the processing of mRNA^{8,9}. DNA sequence analyses of *trans*-recessive intron mutations and characterization of the mitochondrial translation products suggest that *trans*-acting proteins, 'mRNA maturases', involved in splicing are encoded by the two long open reading frames present in the two main introns of the *cob-box* gene,

bI2 and *bI4* (refs 6, 10). The mRNA maturase model of splicing homeostasis can be used as a paradigm of the regulation of split genes in yeast mitochondria^{6,10,11}.

Numerous intron mutations of the *cob-box* gene result in a pleiotropic phenotype: they prevent not only the synthesis of cytochrome *b* but also that of subunit I of cytochrome oxidase. This protein is itself encoded by another mosaic mitochondrial gene, *oxi3*. The lack of expression is due to a block in the correct processing of the oxidase pre-mRNA⁸. More precisely, the fourth intron *bI4* of the *cob-box* gene has been shown to control the expression of *oxi3*^{12,13}. The fourth intron *aI4* of the *oxi3* gene displays a very striking sequence homology to the intron *bI4* but no sequence homology is found between the exons of the two genes¹⁴. There is evidence that the mRNA maturase encoded by the intron *bI4* is involved in excising the two introns *bI4* and *aI4* of the two distant genes¹⁰.

To investigate the function of introns and various components of the mitochondrial splicing machinery, we have developed a general method of selecting and genetically characterizing extragenic suppressors of splicing-deficient mutations located in introns¹⁵. The suppressor *mim2-1* is particularly interesting as (1) it is localized in mitochondrial (mt) DNA; (2) it compensates all the *trans*-recessive *box7*⁻ mutations in intron *bI4*, but does not compensate any of some 450 mutations located elsewhere in the mitochondrial genome; (3) when *mim2-1* is associated with a complete deletion of the *cob-box* gene (leading to a total deficiency of cytochrome *b* and oxidase) oxidase synthesis is partially restored while cytochrome *b* is still absent¹⁶. These observations suggest that in the presence of *mim2-1*, the *cob-box* gene and the mRNA maturase encoded by it are no longer required for splicing and expression of the oxidase gene.

We have now localized the suppressor *mim2-1* in the intron *aI4* of the *oxi3* gene, determined its nucleotide sequence and compared it with that of the wild type. We show here that the suppressor mutation is due to a single base substitution in the intron open reading frame and we suggest that it brings about the synthesis of an active compensating mRNA maturase encoded by intron *aI4*.

Suppressor mutation is on mitochondrial DNA in the oxidase gene

The suppressor mutation *mim2-1* was localized on the mitochondrial genome by using *rho*⁻ deletion mapping according to the first procedure described in ref 15. Three *rho*⁻ (respiratory-deficient mutants that have large deletions of the mitochondrial genome) clones which retained the *mim2-1* mutation were obtained (see Fig. 1) and genetically characterized for the retention or loss of various markers that had previously been mapped in different mitochondrial genes. The *rho*⁻ clones were able to restore, in crosses, the respiratory

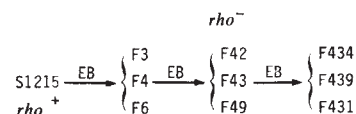


Fig. 1 Genealogy of *rho*⁻ derivatives carrying the suppressor mutation. *rho*⁻ were selected after ethidium bromide (EB) mutagenesis of the revertant *rho*⁺ S1215 and subcloned as described in ref. 15.

sufficiency of two classes of mitochondrial mutations from two distinct regions. (1) They successfully restored all *box7*⁻ mutations of the cytochrome *b* gene at 28 °C and poorly or not at all at 18 °C. This is consistent with the observation that the *mim2-1* suppressor, which they carry, is cryosensitive¹⁵. (2) The clones restored equally well at both 28 °C and 18 °C several *oxi3* mutations of the oxidase gene, showing that they have always retained the suppressor *mim2-1* and several *oxi3* markers which can be introduced by recombination. We conclude that the *mim2-1* mutation is located in or near the *oxi3* gene.

Additional *rho*⁻ subclones with smaller mitochondrial genomes, but still carrying the suppressor, were generated (see Fig. 1). Table 1 represents a simplified genetic map of the *oxi3* gene and the segments of mitochondrial genome retained by the nine *rho*⁻ clones studied. The clones display distinctive genotypes but have retained a coherent region of mtDNA with no internal rearrangements. The retained segments of the *rho*⁻ clones overlap a precise region, located approximately in the middle of the genetic map of the *oxi3* gene, where the *oxi3*⁻ mutations *oxi3-G192* and *oxi3-G2190* are also located¹⁷.

Thus the suppressor *mim2-1* can be localized in the *oxi3* gene near the *oxi3* mutations *G192* and *G2190*.

Suppressor mutation is in the fourth intron of oxidase gene

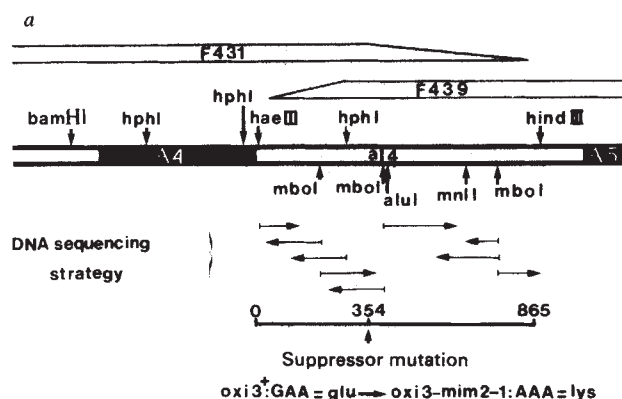
To determine which segment of the *oxi3* gene contains *mim2-1* we prepared mtDNAs of the two overlapping and genetically simple *rho*⁻ clones carrying *mim2-1*, F431 and F439. We performed restriction analyses of the two genomes and compared the results with detailed restriction maps of the wild-type *oxi3* gene^{14,18-20}. The genomes of these two *rho*⁻ clones exist as tandem repeats with unit lengths of 2.2 (F439) and 5 (F431) kilobases (kb). The restriction maps of the two clones are completely consistent with the *rho*⁺ maps already reported. They have retained contiguous segments of mtDNAs with no internal deletions or inversions. The absence of a *Hae*III site in F439 and any *Hind*III site in F431 shows that the overlap between the two genomes spans the segment between the *Hae*III and the *Hind*III sites (Fig. 2). This 865-base pair (bp) fragment contains an internal *Hph*I site present both in F439 and F431. The *mim2-1* mutation can thus be assigned to this

Table 1 Genetic mapping of the suppressor mutation in the oxidase gene

	oxi3 mutants														
<i>rho</i> [−]	G20	G789	G2342	G656	G2133	G2027	G2508	G2190	G192	G3015	G1979	G421	G433	G1683	G922
F3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
F4	−	+	+	+	+	+	+	+	+	+	+	+	+	+	−
F6	−	−	−	+	+	+	+	+	+	+	+	−	−	−	−
F42	−	+	+	+	+	+	+	+	+	+	+	+	−	−	−
F49	−	−	+	+	+	+	+	+	+	+	+	+	+	+	−
F43	−	−	−	−	+	+	+	+	+	+	+	+	+	+	−
F431	−	−	−	−	−	+	+	+	+	−	−	−	−	−	−
F434	−	−	−	−	−	−	−	+	+	+	+	+	+	−	−
F439	−	−	−	−	−	−	−	+	+	+	+	−	−	−	−

rho⁻ clones which have retained the *mim2-1* mutation have been crossed with different *rho*⁺ *oxi3*⁻ mutants; +/− denotes presence/absence of wild-type *rho*⁺ *oxi3*⁺ recombinants in the diploid progeny. As expected from their lineage (Fig. 1), the genomes F42, F43 and F49 are included within the larger genome F4 and the genomes F434, F439 and F431 within the genome of F43. The *oxi3* map is consistent with previous results¹⁷. Strains and genetic methods have been described previously^{15,16}.

Fig. 2 a, Physical mapping of the suppressor mutation in intron *a14* of the oxidase gene and strategy for sequencing. The two upper bars represent the segments of mitochondrial genome retained in *rho*⁻ clones F431 and F439 both carrying the *mim2-1* mutation. The uncertainties due to junction fragments are represented by slanted edges. The middle bar represents the general organization of intron *a14* and flanking exons *A4* and *A5* (solid area). The restriction sites used for physical mapping, cloning and sequencing are shown. The bottom panel represents the DNA sequencing strategy: arrows indicate the direction and approximate length of the fragments sequenced, the restriction sites used for 5'-end-labelling are denoted by a short vertical bar. Nucleotide distances are given from the *Hae*III site (0) located at the beginning of intron *a14*. The mutation *mim2-1* is at position +354. Mitochondrial DNAs were prepared according to ref. 10. A 1,440-bp fragment between the cleavage sites of *Bam*HI and *Hind*III was isolated from the *rho*⁻ genome F43 (Fig. 1, Table 1) and inserted into the plasmid vector pBR322 by *in vitro* recombination following the method described in ref. 24. Plasmid DNAs were prepared according to ref. 25. The recombinant DNA was digested with *Hae*III and *Hind*III and an 865-bp fragment was isolated by agarose gel electrophoresis. This fragment was entirely sequenced by the method of Maxam and Gilbert²⁶.



b

CC CTT ATT ATT ATA TTA ATA ATA TAT AAT GAT ATG CAT TTT TCT AAA TGC TGC AAA TTA TTA AAA AAA TGA ATT ACA AAT ATT ATA AGT CTA TTA TTT AAA
thr ile ile ile leu ile ile tyr asn asp met his phe ser lys cys trp lys leu leu lys lys trp ile thr asn ile ile ser thr leu phe lys

GCC TTA TTT GTA AAA ATA TTC ATA TCT TAT AAT AAT CAG CAG GAT AAG ATA ATA AAT AAT CTT ATA TTA AAA AAA GAT AAT ATT AAA AGA TCC TCA GAG ACT
ala leu phe val lys ile phe ile ser tyr asn asn gln gln asp lys ile ile asn asn thr ile leu lys lys asp asn ile lys arg ser ser glu thr

ACA AGA AAA ATA TTA AAT AAT TCA ATA AAT AAA AAA TTT AAT CAA TGA TTA GCT GGA TTA ATT GAT GGT GAT GGA TAT TTT GGT ATT GTA AGT AAG AAA TAT
thr arg lys ile leu asn asn ser ile asn lys lys phe asn gln trp leu ala gly leu ile asp gly asp gly tyr phe gly ile val ser lys lys tyr

GTA TCA TTA GAA ATT CTA GTA GCA TTA GAA GAT GAA ATA GCT TTA AAA AAA ATT CAA AAT AAA TTT GGT GGT TCT ATT AAA TTA AGA TCA GGT GTA AAA GCT
val ser leu glu ile thr val ala leu glu asp glu ile ala leu lys lys ile gln asn lys phe gly gly ser ile lys leu arg ser gly val lys ala

ATT AGA TAT AGA TTA CTT AAT AAA ACT GGT ATA ATT AAA TTA ATT AAT GCA GTT AAT GGT AAT ATT AGA AAT ACT AAA AGA TTA GTA CAA TTT AAT AAA GTT
ile arg tyr arg leu thr asn lys thr gly ile ile lys leu ile asn ala val asn gly asn ile arg asn thr lys arg leu val gln phe asn lys val

TGT ATT TTA TTA GGT ATT GAT TTT ATT TAT CCA ATT AAA TTA ACT AAA GAT AAT AGT TGA TTT GTT GGA TTT TTT GAT GCT GAT GGT ACA ATT AAT TAT TCA
cys ile leu leu gly ile asp phe ile tyr pro ile lys leu thr lys asp asn ser trp phe val gly phe phe asp ala asp gly thr ile asn tyr ser

TTT AAA AAT AAT CAT CCT CAA TTA ACA ATT TCT GTA ACT AAT AAA TAT TTA CAA GAT GTA CAA GAA TAT AAA AAT ATT TTA GGT GGT AAT ATT TAT TTT GAT
phe lys asn asn his pro gln leu thr ile ser val thr asn lys tyr leu gln asp val gln glu tyr lys asn ile leu gly gly asn ile tyr phe asp

AAA TCA CAA AAT GGT TAT TAT AAA TGA TCC ATT CAA TCA AAA GAT ATA GTA TTA AAT TTT ATT AAT GAT TAT ATT AAA ATA AAT CCA TCA AGA ACA CTA AAA
lys ser gln asn gly tyr tyr lys trp ser ile gln ser lys asp ile val leu asn phe ile asn asp tyr ile lys ile asn pro ser arg thr thr lys

ATA AAT AAA TTA TAT TTA AGT AAA GAA TTT TAT AAT TTA AAA GAA TTA AA
ile asn lys leu tyr leu ser lys glu phe tyr asn leu lys glu leu

*Hind*III-*Hae*III segment in the *oxi3* gene. According to Bonitz *et al.*¹⁴, this fragment is part of the fourth intron *a14* of the *oxi3* gene: the *Hae*III site is located 9 bp downstream from the beginning of the intron and the *Hind*III site is located well inside the intron. The 865-bp fragment carrying the *mim2-1* mutation is therefore entirely within intron *a14*. This physical location is in good agreement with the genetic one, as Netter *et al.*²⁰ have shown by sequence analysis that the mutations *oxi3-G-192* and *oxi3-G2190* are placed inside the intron *a14* and, as discussed above, the *mim2-1* is linked to *G192* and *G2190*. We conclude that the suppressor *mim2-1* is located completely inside intron *a14* of oxidase gene.

Suppressor mutation results from single base substitution in intron open reading frame

We have sequenced the *Hae*III-*Hind*III fragment containing the *mim2-1* mutation according to the strategy described in Fig. 2. This sequence was compared with the corresponding wild-type sequence of the isomitochondrial strain 777-3A (ref. 20). The suppression is due to a single G → A transition (Fig. 3) in the intron open reading frame at position +354 (Fig. 2). This substitution replaces a glutamic acid codon in the wild type by a lysine codon in the suppressor. No other changes occur in the entire sequence.

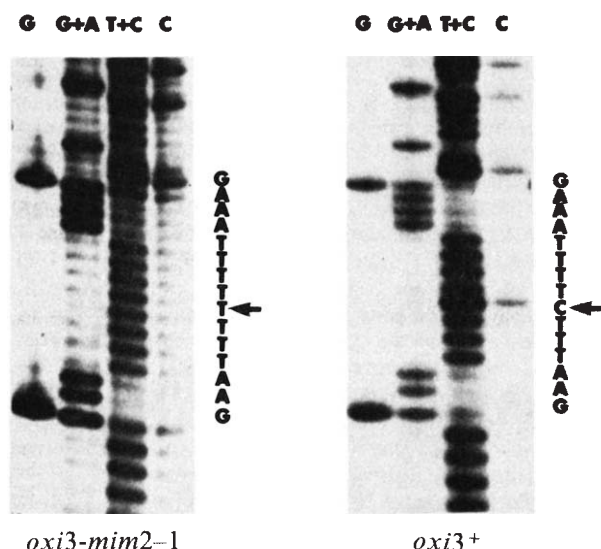


Fig. 3 Sequencing gel demonstrating the base substitution of the suppressor mutation. The mutated *mim2-1* and corresponding wild-type sequences are shown on 8% acrylamide sequencing gels. Both strands of *Mbo*I fragment were sequenced. The base change corresponding to the *mim2-1* mutation that is 36 bp distant from the *Mbo*I site is more visible on the sequence of the nonsense strand. The mutation corresponds to a substitution on the nonsense strand of a C in the wild type by a T in the mutant. On the sense strand this gives a G → A substitution at position +354 (Fig. 2).

Novel mRNA maturase translated from oxidase intron replaced normal maturase translated from cytochrome *b* intron

There is recent evidence that, in the wild type, the splicing of both introns *aI4* of the oxidase gene and *bI4* of the cytochrome *b* gene is controlled by a pleiotropic mRNA maturase translated from *bI4* (refs 10, 20). The activity of this maturase, designated *bI4* maturase, involves the same set of *cis*-acting signals present in both introns, in particular *box9*⁺ and *box2*⁺ nucleotide sequences in *bI4* and their equivalents in *aI4* (see Fig. 4 and

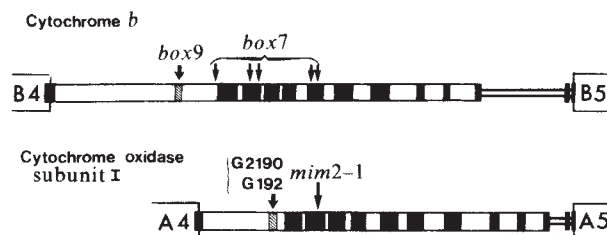


Fig. 4 General organization of the two homologous introns, *bI4* and *aI4*, of cytochrome *b* and cytochrome oxidase genes. *B4*, *B5* and *A4*, *A5* represent respectively the exons of cytochrome *b* and cytochrome oxidase genes. The regions of extensive sequence homology between the introns *bI4* and *aI4* are depicted by solid and hatched segments. Both introns are physically composed of two distinct regions: a long open reading frame (large rectangle) followed by a blocked frame (narrow rectangle)^{10,14,20}. In intron *bI4*, two clusters of *box*⁺ mutational sites, which prevent splicing^{8,9,27}, have been mapped: (1) *box7* cluster composed of *trans*-recessive mutations⁷ which generate nonsense codons in the intron's open reading frame¹⁰; (2) *box9* cluster composed of *cis*-dominant mutations⁷ which are all localized in a dodecanucleotide signal sequence¹⁰. In intron *aI4*, two *cis*-dominant mutations have been localized in the homologous signal sequence (hatched area)²⁰; no *oxi3*⁻ *trans*-recessive mutations have been found until now. The *oxi3-mim2-1* mutation occurs 150 bp downstream from the *cis*-acting signal sequence.

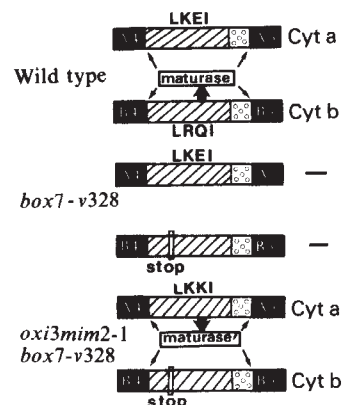


Fig. 5 A model for the normal, deficient and compensatory regulations between the introns of two genes. The drawing represents pre-mRNA molecules transcribed from two genes. The oxidase subunit I (Cyt a) and cytochrome *b* (Cyt b) exons are shown in solid and intron open reading frames in hatched areas. The reading frames are 966 and 1,158 bp long respectively. In the wild type (top), intron *bI4* encodes (thick arrow) a *trans*-acting mRNA maturase which is involved in splicing of both introns, *bI4* and *aI4* (thin arrows). A *box7* mutation (middle) that creates a stop codon at position 405 (AAA → TAA) in *bI4* prevents the synthesis of the maturase and the two introns are no longer excised¹⁰. The unspliced pre-mRNA of *aI4* accumulates and could be translated into a p56 protein devoid of maturase activity (see text). In a double mutant carrying the *oxi3-mim2-1* mutation (bottom), the protein translated from *aI4* has acquired the maturase activity due to the replacement of the glutamic (E) by the lysine residue (K). This novel protein permits splicing and expression of both genes.

refs 10, 20). We propose that the *mim2-1* mutation induces the synthesis of an active *aI4* maturase that would not occur in the wild type and that it is translated from the oxidase intron. This novel maturase would act as a substitute for a deficient *bI4* maturase, using the same set of critical sequences present in both pre-mRNAs. Such a hypothesis (summarized in Fig. 5) explains all the known facts: the suppression by *mim2-1* of *bI4* maturase-deficient mutants and the absence of suppression of *box9*⁻ or *box2*⁻ mutants; the synthesis of oxidase in the presence of a complete deletion of the cytochrome *b* gene¹⁶; and sequence homology between *aI4* and *bI4* (refs 10, 14, 20).

How is a new maturase formed from an inactive intron?

One could imagine, for example, that, in the wild type, intron *aI4* is not translated at all because an internal splice destroys the continuity of its open reading frame; the *mim2-1* mutation would remove the internal splice point and modify the splicing pathway such that the continuity of the reading frame would be restored. It might also be possible that, in the wild type, intron *aI4* is translated but the protein is destroyed by proteolysis; the *mim2-1* mutation could have removed a specific proteolytic site. Alternatively, the wild-type protein could be inactive and the *mim2-1* mutation, by creating a proteolytic site, would permit the formation of an active fragment involved in splicing. We find these conjectures unattractive, however, because they do not explain why an open reading frame is preserved in the wild-type oxidase intron *aI4*. We therefore favour the hypothesis that *aI4* is normally translated into a protein. If this is the case, what is its function in the wild type, why is it not *trans*-active in splicing (while *bI4* is) and how does *mim2-1* restore the splicing activity?

We believe that the ability of *mim2-1* to restore splicing activity is due to a local increase of positive charges in a part of a protein that is essential for its catalytic activity. Arguments favouring this idea are: (1) *mim2-1* causes a replacement of a glutamic codon by a lysine codon and thus increases the charge

by +2. Interestingly, this change occurs in an α -helical region as shown by our calculation of the secondary structure carried out according to ref. 21 (data not shown). This α -helix is the longest (15–18 amino acids) that can be traced in the translation products of introns *bI4* and *aI4*, and occupies homologous positions in both proteins. The α -helix of the wild-type *bI4* translation product (active) is more basic than that of the wild-type *aI4* product (inactive) by two or four charges (depending on the local polymorphism between the strains D273–10B and 777–3A)^{10,20}. The *mim2-1* mutation, by increasing the charge by +2, renders the *aI4* translation product more similar to its normal *bI4* counterpart and we believe that this change is sufficient to explain the acquisition of splicing activity. That a protein which interacts with nucleic acids should be positively charged is plausible, and other active mRNA maturases, such as that of *bI2*, are overall highly basic^{6,13}.

(2) A second line of evidence favouring the idea that *aI4* can be translated even in the absence of *mim2-1* mutation comes from the analysis of mitochondrially synthesized proteins in splicing-deficient mutants resulting from *bI4* maturase-deficient mutation or *oxi3*⁻-G192 and -G2190 mutations in *aI4* (O. Groudinsky, personal communication). These mutants accumulate a protein of apparent molecular weight 56,000 (referred to as p56). We conjecture that p56 results from translation of the previously fused oxidase exons A1–A4 with the *aI4* open reading frame. The size of p56 is consistent with this idea and fingerprint comparisons show that p56 displays some peptide homologies with the subunit I of cytochrome oxidase²². Analyses of double mutants indicate that the N-terminal sequences of the *oxi3* exons control the synthesis of p56 (O. Groudinsky, unpublished results) and this protein disappears in an *oxi3* deletion mutant²³. All these data are easily explained by the model shown in Fig. 5: in the wild type, splicing of *oxi3* mRNA is efficient and no p56 accumulates because the *oxi3* pre-mRNA does not accumulate. In mutants deficient for the *bI4* maturase (*box7*⁻ mutants¹⁰) or *cis*-dominant *aI4* mutants, such as *oxi3*⁻-G192 (ref. 20), the pre-mRNA containing the non-excised intron *aI4* would accumulate and its translation would lead to an accumulation of p56. In a double mutant carrying simultaneously a *box7*⁻ mutation and a *mim2-1* mutation, a p56 protein should still be detectable because the suppression is only partial, probably because the *aI4* maturase is less efficient than its normal *bI4* counterpart; this has indeed been observed¹⁶. Our model predicts that p56 from an *oxi3*⁻ *mim2-1* strain should differ slightly from the *oxi3*⁺ in its fingerprint pattern, and preliminary experiments (O. Groudinsky *et al.*, unpublished results) suggest that this is indeed the case.

Evolution of two homologous introns in two non-homologous genes

Sequence homologies between *bI4* and *aI4* (refs 10, 14, 20) and functional compensations between their translation

products (this work) strongly suggest that the two introns are derived from a common ancestral sequence. We do not know when this duplication, transposition or insertion took place, but depending on whether it is recent or ancient even, two evolutionary histories can be proposed. If it is a recent event, then the fact that *aI4* still has an open reading frame could be purely accidental. The *bI4* descendant of the common ancestor would have kept the splicing function and the *aI4* descendant would have lost it by accumulating missense mutations. The divergence would not have been large enough to prevent a single base substitution, such as in *mim2-1*, still being able to restore or readjust the function. In other words, *aI4* may have just become (or be in the process of becoming) a pseudogene and we have caught it during the very early part of its evolutionary drift! Although this hypothesis cannot be formally excluded, we do not find it plausible and prefer to think that the duplication, transposition or insertion event took place a long time ago. We propose the following evolutionary scheme.

An identical ancestral base sequence, encoding a protein, was originally present or has been inserted, in two non-identical genes. This sequence has diverged during evolution in two directions: the first one has led to a diffusible protein endowed with a pleiotropic splicing activity (the *bI4* sequence) and the second to a protein with no catalytic splicing activity (the *aI4* sequence). Numerous missense mutations have been fixed during this period: for the region of strongest homology between *aI4* and *bI4*, which is 170 residues long, some 38–40 amino acid replacements have taken place^{10,14,20}. With such an amount of evolutionary divergence there must be a selective pressure that has prevented the accumulation of nonsense mutations in *aI4*. What is the role of *aI4*? One hypothesis is that the normal translation product of *aI4* has a different and unknown function. It could, for example, be a structural part of the mitochondrial splicing and/or translation apparatus, it could be used as a handle to position correctly, by quaternary interactions, the *bI4* maturase on its second substrate, that is, on the pre-mRNA of oxidase. Alternatively, the travelling of a ribosome through *aI4*'s open reading frame could be a necessary but insufficient condition for the processive splicing (see refs 10, 20). In both hypotheses, the splicing of the two genes will be catalysed by a single maturase and not by two independent ones. This could confer a selective advantage because a coordinate expression of both genes would be facilitated.

Evolutionary conjectures are not easy to test. The finding of a converse situation to the normal one and the demonstration that it results from a single base change in an intron do, however, provide a striking example of intergenic communications through intron-encoded proteins. It is thus of interest for the evolutionary history of mosaic genes.

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LETTERS TO NATURE

Is our vacuum metastable?

Michael S. Turner

Astronomy and Astrophysics Center, The University of Chicago,
Chicago, Illinois 60637, USA

Frank Wilczek

Institute for Theoretical Physics, University of California,
Santa Barbara, California 93106, USA

In spontaneously broken gauge theories of particle interactions there are sometimes several local minima of the effective potential. Any of these minima can serve as a vacuum in the sense that we can expand the fields around their values at the minimum, interpret the quantized fluctuations around the minimum as particles, and compare the properties of these particles with experiment. One might think that only the state with absolutely minimum energy could be what we ordinarily call our vacuum, as the other local minima will inevitably decay into this lowest one. However, this is not necessarily the case, because it is possible for the lifetime of a metastable vacuum to be very long, even when compared with the age of the Universe. Furthermore, the energy densities available in present laboratory or astrophysical environments are much less than the barriers which exist in field space between the different local minima, and so we have no direct sensitivity to the presence of a possibly lower vacuum state. (A possible exception to this are magnetic monopoles which do probe the large field region.) If it is not the absolute minimization of the effective potential, what does determine the present vacuum state of the Universe? We argue here that it is determined cosmologically by the dynamical evolution of the Universe from the hot, dense phase which existed shortly after the big bang to the present^{1,2}.

At finite temperature, the free energy tends to be minimized in phases which contain light particles. Therefore, we expect that in general, at extremely high temperatures the thermodynamically favoured phase is the symmetric one which contains the most massless particles. Assuming that very near the big bang this phase is realized³⁻⁵, one can follow the subsequent dynamical evolution to determine the present state. The present vacuum is properly determined by evolutionary, not merely energetic, considerations. This point is relevant for all proposed models of elementary particle interactions.

In what conditions might we convince ourselves that we live in a metastable vacuum (or, not finding these conditions, assure ourselves that we do not)? Of course, we must demand that the putative vacuum has an extremely long lifetime, and that small fluctuations around it describe known physics. In addition, it should have been realized in the dynamical evolution of the Universe, starting from the symmetric phase at extremely high temperatures.

First, consider the question of metastability. For a ϕ^4 scalar field theory in which the energy difference between the two minima is not too large, the decay rate of the 'false vacuum' (per unit volume per unit time) at zero temperature is⁶

$$\Gamma \approx A \exp[-(32\pi^2/(3\epsilon^3\lambda))] \quad (1)$$

where λ is the quartic self-coupling, ϵ is the ratio of the energy difference between the two minima to the barrier height between them, and A has dimensions of mass⁶ and should be $O(\mu^4)$. Here μ^2 is the bare mass of the scalar field. Taking μ to be of the order of the grand scale ($\sim 10^{14}$ GeV), and the spatial volume to be that of the present observable Universe

($V \sim 10^{84}$ cm³), the probability that an energetically less favourable minimum has decayed during the age of the Universe is exponentially < 1 so long as

$$\epsilon \ll 0.6 \lambda^{-1/3} \quad (2)$$

a condition which is easily satisfied.

We now examine the second and third issues in the context of the simplest unified model of particle interactions, the minimal SU(5) theory⁷.

At zero temperature the effective potential can be written as^{8,9}:

$$V(h, A) = \frac{-\mu^2}{2} \text{tr} A^2 + \frac{a}{4} (\text{tr} A^2)^2 + \frac{b}{2} \text{tr} A^4 \\ - \frac{\nu^2}{2} h^\dagger h + \frac{\lambda}{4} (h^\dagger h)^2 + \alpha (h^\dagger h) \text{tr} A^2 + \beta h^\dagger A^2 h \quad (3)$$

where the discrete symmetry $A \rightarrow -A$ has been imposed, h is an SU(5) vector **5** Higgs field, and A is an SU(5) adjoint **24** Higgs field (traceless hermitian matrix). To reproduce the standard (and thus far very successful) SU(5) phenomenology, there must be a local minimum where A acquires a large vacuum expectation value of the form,

$$\langle A \rangle = \begin{pmatrix} 1 & & & & 0 \\ & 1 & & & \\ & & 1 & & \\ & & & -3/2 & \\ 0 & & & & -3/2 \end{pmatrix} \delta \quad (4)$$

This vacuum expectation value breaks SU(5) down to SU(3) $\times$ SU(2) $\times$ U(1).

The second stage of symmetry breaking involves the fifth component of h acquiring a vacuum expectation value much less than δ (the small vacuum expectation value $\langle h \rangle$ will slightly perturb $\langle A \rangle$ from the form given in equation (4); see refs 8-10). In addition, we must also ensure that the colour triplet component of h has a large mass $\approx 0(\delta)$ as it can mediate proton decay. Both of these requirements are fulfilled if:

$$\beta < 0 \quad (5a)$$

$$-m_{\text{eff}}^2 \equiv \nu^2 - 3\mu^2(10\alpha + 3\beta)/a' \approx 0 \quad (5b)$$

$$(\alpha + \beta/2) > -[\lambda(a+b)]^{1/2}/2 \quad (5c)$$

$$\mu^2, a', b > 0 \quad (5d)$$

$$\lambda > 9\beta^2/10b \quad (5e)$$

where $a' \equiv 15a + 7b$. Condition (5b) specifies that the SU(2) doublet component of h (the ordinary Weinberg-Salam doublet) has a small, negative bare mass. Quantitatively, m_{eff} must be about 13 orders of magnitude smaller than $\delta = (2\mu^2/a')^{1/2}$.

For our purpose, the crucial observation is that condition (5b), along with the others, can be satisfied with ν^2 large and positive. Looking back at equation (3), it is perhaps not surprising that in this case another very different type of vacuum might be energetically favourable: one in which $\langle h \rangle$ is large. Indeed, if

$$2\alpha\nu^2/\lambda > \mu^2 \quad (6a)$$

$$2(\alpha + 4\beta/5)\nu^2/\lambda > \mu^2 \quad (6b)$$

then the potential also has a local minimum at $\langle h_s \rangle^2 = \nu^2/\lambda$ and $\langle A \rangle = 0$. In this vacuum SU(5) has broken down to SU(4). When we compare the value of the effective potential at this local minimum with the usual one (when both exist), we find that for the following range of parameters:

$$\mu^2, \lambda, a', b, -\beta > 0 \quad (7a)$$

$$\lambda > 9\beta^2/10b \quad (7b)$$

$$(\alpha + \beta/2) > -[\lambda(a+b)]^{1/2}/2 \quad (7c)$$

$$(5\alpha + 4\beta)(10\alpha + 3\beta) > 5\lambda a'/6 \quad (7d)$$

$$\nu^2 \approx 3\mu^2(10\alpha + 3\beta)/a' \quad (7e)$$

the usual vacuum corresponds to a local, but not a global, minimum.

From the point of view of microphysics, then, it is quite conceivable that our vacuum is metastable. We must now investigate the dynamical question, whether it is reasonable that during the course of its temperature evolution, the Universe could get 'hung up' in our vacuum in spite of its only being metastable. Basically, we must determine which of the two asymmetric vacua becomes lower than the fully symmetric vacuum first as the Universe cools down from very high temperatures. Although we have not investigated this question in quantitative detail, it seems that a 'hang up' can occur. Finite-temperature modifications to the zero-temperature potential

tend to increase the free energy, and those due to the most strongly coupled fields are most important³⁻⁵. Suppose that the usual vacuum and the SU(4) vacuum discussed above have roughly the same energy at zero temperature. The high temperature state continuously related to the SU(4) vacuum will then be disfavoured if it feels stronger finite-temperature corrections. This will occur, for instance, if all the Higgs self-couplings are small and $\hbar$, but not A , couples strongly to some fermion field.

It seems distinctly possible in simple unified models, that our present vacuum is only metastable, and that nevertheless, the Universe would have chosen to get 'hung up' in it. If this is the case, then without warning, a bubble of true vacuum could nucleate somewhere in the Universe and move outwards at the speed of light, and before we realized what swept by us our protons would decay away. There is no immediate cause for concern, however, because the lifetime of our metastable vacuum could easily be 10^{30} yr (or only 10^{10} yr for that matter).

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A search for evidence of superheavy-element fission in chondritic metal

R. K. Bull*‡ & P. Mold*†

* Department of Physics, University of Birmingham B15 2TT, UK

† West Midlands College of Higher Education, Walsall, West Midlands, UK

There is evidence for the early melting of the Moon^{1,2}, and Runcorn²⁻⁴ has concluded from palaeomagnetic data that a fluid core dynamo must have been present for $\sim 10^9$ yr and that the decay of a siderophile superheavy element (SHE) with a half life of $\sim 10^8$ yr may have been responsible for these phenomena³⁻⁵. Libby and co-workers⁶ have suggested that SHE-decay could have produced certain trace-element abundance patterns in iron meteorites. Several searches⁷⁻⁹ have failed to detect evidence for SHE decay in iron meteorites. We describe here a search for SHE in metal fragments within ordinary chondrites. The absence of excesses of tracks along the boundaries between olivines and metal grains allows upper limits to the SHE content of the metal of $\sim 2.5 \times 10^{-13}$ to $\sim 2.5 \times 10^{-15}$ kg per kg to be set at a time $\sim 4 \times 10^9$ yr ago, determined from the ^{244}Pu fission track densities in chondritic phosphates.

Our experimental methods are essentially similar to those described previously⁷. Slices and coarse fragments from three chondrites, Estacado (type H6), Wold Cottage (L6) and Alta'ameem (LL5), were ground and polished. They were then etched in WN solution at pH 8 and 50% of the usual concentration to reveal tracks in olivine crystals¹⁰. The etched samples were then examined under an optical microscope at a magnification of approximately $\times 630$ and under a scanning electron microscope at about $\times 5,000$ magnification. Abundant cosmic ray tracks (identification as cosmic ray tracks was based on the anisotropic angular distribution of the tracks and a length distribution peaking at short track lengths) were observed

throughout the olivine grains from Alta'ameem but track densities were observed to be much lower in Wold Cottage and Estacado (see Table 1). The boundaries between the olivine crystals and metal grains were carefully examined using both scanning electron microscopy and optical microscopy, and no significant excesses of tracks were detected in these regions in any of the three meteorites. For the purposes of calculation it is assumed that the contribution of fission tracks from the metal is, in each case, equal to the cosmic ray track density. These track densities (column 4 of Table 1) allow upper limits to the SHE content of the metal to be calculated and these are displayed in column 5 of Table 1. In obtaining these estimates it is assumed that the SHE have decayed to extinction during the time since tracks began to be retained by the olivine. These limits apply to the times at which track retention in the olivine began for each of the three meteorites and for any conclusions to be drawn from these data it is necessary to establish a time scale for track retention by the olivine.

An estimate of the track retention time for these meteorites can be made by examining the fission track densities in apatite and merrillite crystals from each meteorite. These phosphate crystals are usually found to contain tracks produced by the fission of ^{238}U and ^{244}Pu . Since the latter has a half life of $\sim 10^8$ yr, the identification of ^{244}Pu tracks in these phases would provide strong evidence that track retention in the olivine occurred relatively close to the time of formation of the meteorites, because olivine is believed to retain tracks at higher temperatures than apatite and merrillite¹¹.

Phosphates were concentrated in non-magnetic fractions of density between 2.8 and 3.3 g cm⁻³ and grain size between 63 and 106 μm . These were then etched in aqueous HNO_3 solutions at room temperature. The precise etching conditions used in each case are shown in Table 2. The track densities were obtained from scanning electron microscopy photographs at a magnification of about $\times 5,000$. The uranium concentrations in the phosphates were determined by irradiating them with thermal neutrons (fluences of about 10^{18} neutrons cm⁻²) in the Herald reactor at Aldermaston. Induced-fission fragments were detected in adjacent sheets of uranium-poor mica. The neutron fluence was monitored by irradiating standard reference glasses along with the meteorite samples.

Fission track density and uranium content data are shown in Table 2. The track density figures for Alta'ameem have been

‡ Present address: Atomic Weapons Research Establishment, Aldermaston, Reading, Berks RG7 4PR, UK.

Table 1 Track densities in olivines

Meteorite (class)	Central track density in olivines* (cm ⁻²)	Track density along boundary with metal† (cm ⁻²)	Upper limit to contribution from fission in metal (cm ⁻²)	Upper limit to SHE concentration at time of track retention by olivine (kg per kg)
Wold Cottage (L6)	<10 ⁴	<10 ⁴	10 ⁴	2.5 × 10 ⁻¹⁵
Alta'ameem (LL5)	(0.9–1.3) × 10 ⁶	~10 ⁶	10 ⁶	2.5 × 10 ⁻¹³
Estacado (H6)	<10 ⁴	<10 ⁴	10 ⁴	2.5 × 10 ⁻¹⁵

* Mainly due to heavy cosmic ray primaries.

† Measured within a strip of olivine 10 µm wide immediately adjacent to metal grains.

Table 2 Summary of track data for phosphates

Meteorite	Mineral	Etching conditions	Track density† (10 ⁶ cm ⁻²)	U content (10 ⁻⁶ kg per kg)	Approximate average ratio of ²⁴⁴ Pu fission tracks to ²³⁸ U fission tracks
Wold Cottage	Merrillite*	75s, 0.25% HNO ₃ , 20 °C	67–82 (6)	0.08–0.19	165
	Apatite		16–25 (2)	1.8–3.7	2
Alta'ameem	Merrillite	20s, 1% HNO ₃ , 20 °C	34 (1)	0.1	100
	Apatite		7–16 (20)	0.3–1.7	4
Estacado (This work)	Merrillite	50s, 1% HNO ₃ , 20 °C	33 (18)	0.1	120
(Pellas and Storzer ¹⁵)	Merrillite		37.5 (15)	0.19	75
	Apatite		8.2 (15)	3.26	<1

* In many earlier papers on meteoritic phosphates, this mineral is referred to as whitlockite.

† Number of crystals shown in parentheses.

corrected for a cosmic ray track background of ~10⁶ cm⁻². The contribution to the total track density from the spontaneous fission of ²³⁸U can be calculated from the measured uranium content of each phase. The excess fission tracks are assumed to be due to the spontaneous fission of ²⁴⁴Pu and the last column of Table 2 gives the ratio of ²⁴⁴Pu to ²³⁸U fission tracks. The assumption of a ²⁴⁴Pu source for these tracks rests on numerous determinations of ²⁴⁴Pu fission Xe in merrillites^{12–14} (formerly known as whitlockites) and in apatites¹³ from chondritic meteorites. In particular, Kirsten and co-workers¹³ have measured the fissionogenic Xe content of Estacado. The fission track data for Estacado obtained by Pellas and Storzer¹⁵ is included in Table 2.

Merrillites from all three of the meteorites that we studied show large excesses of ²⁴⁴Pu fission tracks and notwithstanding uncertainties in the initial Pu content of these crystals, it seems unlikely that these three meteorites could have cooled to the track retention temperature of merrillite (and therefore of olivine) much later than ~4 × 10⁹ yr ago. The upper limits to SHE concentrations given in Table 1 therefore apply to approximately this time. Runcorn⁴ requires a SHE concentration in the lunar core of ~10⁻⁷ kg per kg at ~4.1 × 10⁹ yr ago. The concentration would have fallen to ~6 × 10⁻⁹ kg per kg at ~3.7 × 10⁹ yr ago, assuming a SHE half life of ~10⁸ yr. Our results indicate that the SHE content of chondritic metal was lower by at least 4–6 orders of magnitude at that time.

These results, taken together with the failure to find evidence for SHE in the metal from iron meteorites^{7–9}, indicate that the abundance of SHE in meteoritic metal was very low ~4 × 10⁹ yr ago and unless the metal of the lunar core was very much enriched in siderophiles relative to meteorites, SHE could not have played an important role in the early heating of the Moon.

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Detection of nitrogen at {100} platelets in diamond

S. D. Berger & S. J. Pennycook

Cavendish Laboratory, Madingley Road, Cambridge CB3 0HE, UK

Platelets in type Ia diamond were first directly observed with transmission electron microscopy by Evans and Phaal¹ in 1962. Considerable controversy still exists over their structure and chemical composition. One of the earliest models of the platelet structure was that proposed by Lang² in which nitrogen, the major impurity in type Ia diamonds, is segregated into plate-like structures, only two atoms thick, lying on the cube planes. Later evidence seemed to favour interstitial carbon as the major component of the platelets^{3–5}, although more recent evidence from the production of platelets in synthetic diamond⁶ again points to nitrogen as forming an important constituent. High resolution electron microscopy also supports this view^{7,8}. Here we report for the first time the direct observation of nitrogen at platelets using the technique of electron energy loss spectroscopy (EELS).

A type Ia diamond was cut and thinned parallel to an (001) plane. The platelets were studied using a VG Microscopes' HB5 scanning transmission electron microscope (STEM). In the STEM transmitted electron images showing diffraction or phase contrast can be obtained similar in all respects to those obtained in a conventional TEM, but in addition elemental analysis and diffraction patterns can be obtained at very high

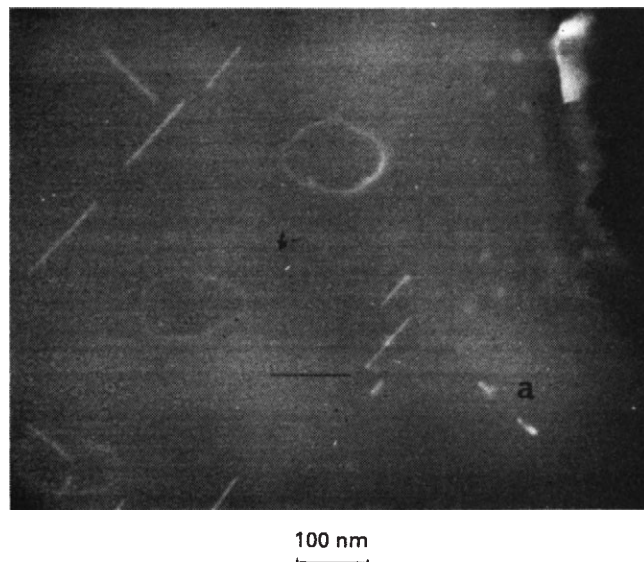


Fig. 1 Weak beam image of platelets in type Ia diamond. The dark marks are burns in the phosphor of the display tube.

spatial resolution (see ref. 9 for a full discussion of the techniques). Figure 1 shows an edge of the diamond flake containing several platelets. The image is obtained using an annular dark field detector, which detects electrons scattered out of the main beam. The diamond is orientated with the electron beam incident exactly along the [001] direction, so that the image is formed mainly from the four 220 Bragg reflections, weakly excited. Three sets of platelets are visible. Those lying on the (100) and (010) planes are seen as two sets of straight lines perpendicular to each other, while those lying on the (001) plane within the foil have a roughly circular shape, made visible by the partial dislocation around the circumference of the platelet. In thinner regions of the diamond, platelets lying parallel to the beam may extend through the thickness of the specimen (platelet a in Fig. 1 for example). In such regions the diffraction contrast along the length of the platelet is lost but it is still visible at the ends where the defect terminates inside the crystal. The central region of such platelets can be imaged by phase contrast microscopy giving images similar to those shown by Bursill *et al.*⁸, although the resolution is limited to about 0.7 nm, in our case, due to stray magnetic fields around the field emission gun.

A previous attempt¹⁰ to find nitrogen by EELS failed due to insufficient sensitivity to nitrogen in the experimental conditions used. Several factors must be optimized to achieve the highest sensitivity when analysing a platelet with a fine electron probe. First, steps must be taken to prevent the build-up of carbon contamination on the area analysed. This can be a severe limitation to microanalysis at the highest spatial resolution, but was completely eliminated by baking both the microscope and

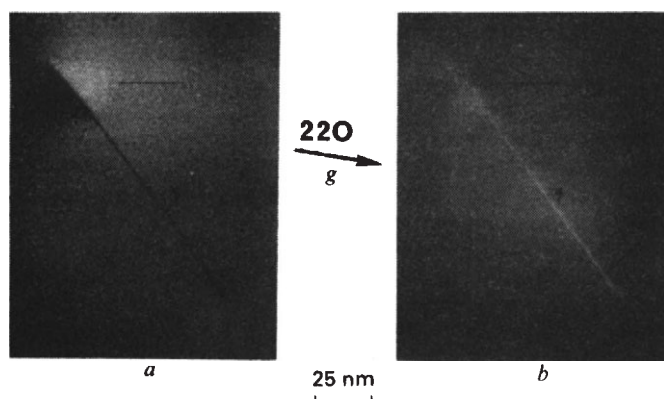


Fig. 2 Typical two-beam bright field (a) and annular dark field (b) images of platelets suitable for microanalysis.

specimen at 120 °C for 10 h before analysis. Second, the specimen geometry was optimized by choosing platelets parallel to the beam running right through the thickness of the diamond. The highest sensitivity to the elemental composition is then achieved by passing the smallest electron probe down the centre of the platelet. The third factor is the correction for electron beam or specimen drift. As type Ia diamond is an insulating material, drift tends to occur as the specimen charges up and it is important to keep the electron probe over the platelet throughout the analysis. By scanning the smallest possible area ($\sim 1 \text{ nm}^2$) an image is available on the annular dark field detector while the electrons in the bright field beam are analysed by the spectrometer. In this way drift can be continuously compensated for using electrical beam shift controls. To facilitate this correction, improved contrast in the annular detector image was obtained by tilting the diamond slightly off the [001] direction into a two-beam condition, typical images being shown in Fig. 2.

The fourth and possibly most important factor is to achieve the most accurate background subtraction under the nitrogen edge in the energy loss spectrum. This would normally be done by extrapolation from the region of the spectrum just before the edge. However, due to strong intensity modulations following the carbon K-edge this technique would be inaccurate in this case. The spectrum from the normal diamond lattice shows

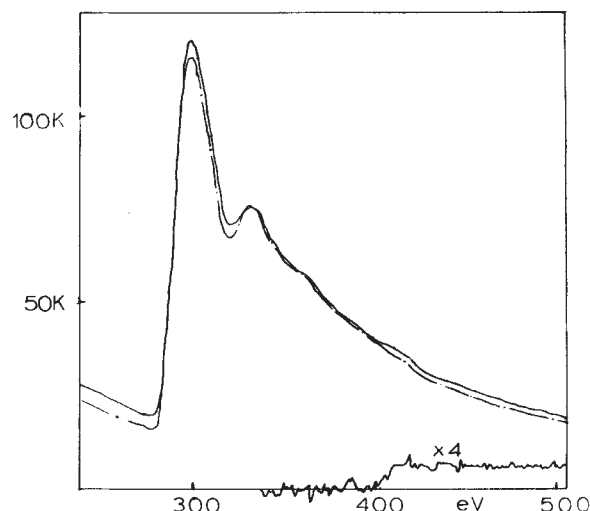


Fig. 3 Electron energy loss spectra from the centre of a platelet (solid line) and from just off the platelet (broken line). Also shown is the difference ($\times 4$) which shows the nitrogen-K edge at $\approx 406 \text{ eV}$ from the platelet.

the same modulations and hence provides a much more accurate background for subtraction.

The spectra were obtained by scanning an isolated drift tube to give an absolute energy calibration¹¹ with digital data acquisition using a Link Systems 860 analyser. The incident beam semi-angle $\alpha = 8.3 \text{ mrad}$ and spectrometer acceptance semi-angle $\beta = 3 \text{ mrad}$ were chosen to give good spatial resolution together with a high count rate in the spectrometer with an energy resolution of about 12 eV.

The spectrum from the platelet acquired in this way is shown in Fig. 3 and is compared with the spectrum taken from the diamond just adjacent to the platelet. As well as the carbon K-edge at $\approx 290 \text{ eV}$ an edge is clearly visible at $\approx 406 \text{ eV}$ from the platelet which corresponds to the nitrogen K loss (both edges are shifted by $\sim 7 \text{ eV}$ from their expected positions¹² due to band structure effects¹³). The mismatch between the spectra in the region of the carbon edge is due to a slight change in specimen thickness between the two areas analysed. The area of normal lattice analysed was slightly thicker due, possibly, to preferential etching of the platelet during preparation. Generally, the effect of taking a matrix spectrum from a thicker region would be to decrease the slope of the decaying carbon edge as a result of multiple scattering. This would manifest

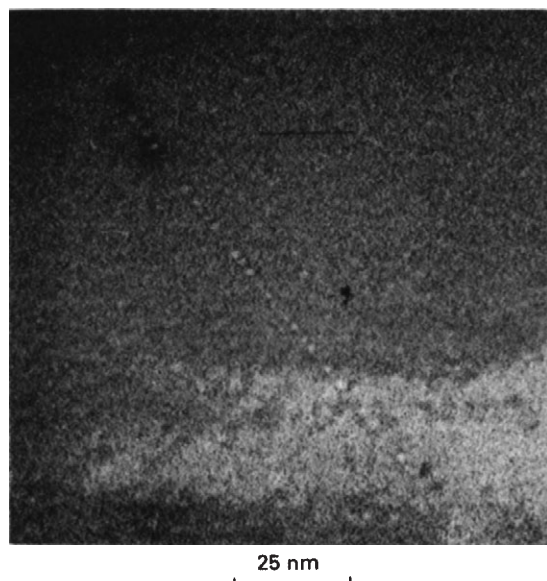


Fig. 4 Bright field image of an inhomogeneous platelet, or 'voidite' (noisy image is due to a shorter exposure time than in Figs 1 and 2).

itself, after subtraction from the platelet spectrum, in a slow modulation on the difference curve. In our case the change in thickness is ~3% and, as can be seen, produces negligible modulation of the difference between the spectra. The feature at ~406 eV rises over ~12 eV, corresponding to the spectrometer resolution, continues for the rest of the spectrum and can only be due to a nitrogen *K*-absorption edge. It has been shown¹⁴ that most of the nitrogen in type Ia diamonds is in a dispersed form. Any signal due to this nitrogen will appear in both the background spectrum and the spectrum from the platelet. Thus when the two spectra are subtracted, only the nitrogen signal associated with the platelet will remain. However, the concentrations of the dispersed forms of nitrogen are so low that it is unlikely that they will be detected at all by this technique. The result of subtracting the two spectra is shown on an increased vertical scale and the nitrogen edge is very clearly visible.

So far, five platelets have been analysed, of which four contained nitrogen to a significant extent, while one contained no detectable nitrogen (<10% of the amounts found in the other platelets). By scanning an accurately known area and integrating the counts under the carbon and nitrogen edges in the spectrum, it is possible to obtain the percentage of nitrogen present in the volume analysed, and this can be compared with the expected percentage of nitrogen present if the platelet followed the Lang model. We found that the percentage of nitrogen detected varied significantly and no platelet contained as much nitrogen as the model predicts. Sequential spectra were then taken from the same platelet and it was found that nitrogen was being gradually lost from the defects. Although this would appear to account for the variation in nitrogen content already seen, early results indicate that some platelets are more susceptible to damage than others and further experiments are being carried out to clarify this point.

One platelet was found which showed a particularly interesting form of inhomogeneous contrast and is shown in Fig. 4. Analysis showed the bright regions of Fig. 4 contained at least three times as much nitrogen as the dark regions. The contrast seen from this platelet is very similar to that seen from 'voidites' (T. Evans and J. Macguire, and J. L. Hutchinson, personal communications) which appear to be a form of collapsed or semi-collapsed platelet. If the bright regions of Fig. 4 do not extend through a significant fraction of the thickness then they must contain a very high concentration of nitrogen.

The results from this work show directly that nitrogen is associated with platelet defects in type 1a diamond. The fact

that some platelets are susceptible to beam damage at an energy of 80 keV indicates perhaps that the nitrogen is not bonded into the lattice in the covalent manner that the Lang model suggests. Also, if beam damage is unable to account for the variable nitrogen content seen, then again a modification to the simple models is called for.

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Reconstruction of past ice conditions in a Hudson Bay estuary using tree rings

Gordon C. Jacoby & Linda D. Ulan

Lamont-Doherty Geological Observatory, Columbia University, Palisades, New York 10964, USA

Descriptions of ice conditions, ranging from lake and sea ice to glacial advances and retreats, form an important portion of the climatic record. Ice formation, deterioration and changing areal extent are of increasing interest in climatology, sea transportation, petroleum exploration and the fishing industry. With few exceptions, quantitative information on ice conditions is generally brief, with many records in the US and Canada beginning only in the 1950s. Tree-ring data from long-lived trees can be used as quantitative proxy data for past climatically related parameters. The record of first complete freezing of the Churchill River estuary on Hudson Bay is reconstructed here for 298 yr (1680-1977) by means of tree-ring analysis.

Both high-latitude tree growth and ice are influenced by many factors but can be significantly related to temperature²⁻⁵. In areas where ice conditions are influenced by a complex set of factors, a simple or direct relationship with climate may be difficult to establish. However, ice conditions in some areas show a more direct relationship to particular climate parameters (P. M. Kelly, personal communication). It is in these latter areas that we are investigating the possibility of using selected tree-ring data as a proxy series for past ice conditions.

A promising location to test the feasibility of reconstruction is near Churchill, Manitoba, Canada (see Fig. 1). Both a recent record of ice conditions and an independent, historical record are available, and we have developed a tree-ring chronology from the same location. The historical record of ice conditions is now used to calibrate the tree-ring time series which is in turn used to reconstruct the ice conditions for almost three centuries. The reconstruction is verified by modern data.

The tree-ring chronology is based on annual ring-width measurements of 15 increment cores from 7 white spruce (*Picea glauca* [Moench] Voss) from flat, poorly drained muskeg overlying permafrost. Ten of the 15 cores extend back to 1686 or

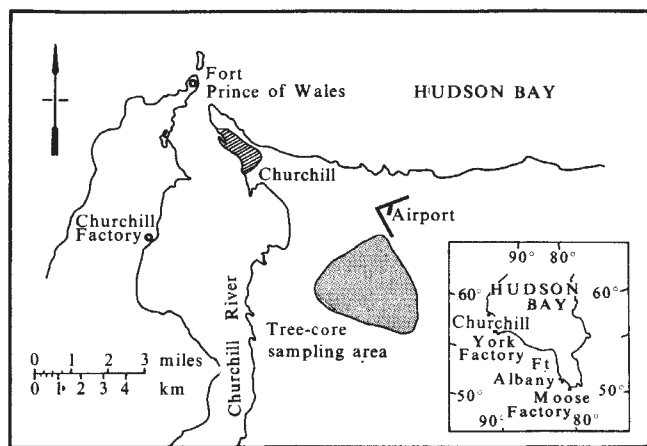


Fig. 1 Map of the Churchill area showing tree-core sampling site, Fort Prince of Wales, airport (meteorological station) and modern Churchill. Although total collection at this site was 39 cores from 15 trees, we feel that objective screening out of cores with abnormal anatomy (mainly reaction wood—eccentric growth due to leaning) and use of principal component analysis (PCA) to determine the most homogeneous subgroup produces a chronology with a better climatic signal-to-noise ratio. The PCA procedures are used before and independently of comparisons between the tree-ring data and meteorological information. The purpose is to determine a subgroup of tree cores with maximum common variance, which presumably is dominated by climate. The PCA results in ref. 20 show that, even from one site, smaller, homogeneous subgroups of cores can produce equally good or better climatically sensitive time series than larger numbers of cores. Cross-spectral analysis has also been used in selecting coherent subsets of cores from sites in Switzerland to maximize the signal-to-noise ratio of the resulting chronologies (J. Guiot, personal communication). Because we calibrated with historical data from 1741 to 1764, we also deleted cores younger than 1741 so that the sample size would not change between the calibration and verification periods. The deletions proceeded as follows: 17 cores due to abnormal growth or extreme youth of tree, 3 cores (all from one tree) shown by PCA to be heterogeneous with respect to the common variance of the rest of the cores, and 4 cores younger than 1740. Due to heterogeneous low-frequency variations in ring widths, conventional standardization methods did not produce a usable time series of ring-width indices for these tree cores. The s.d. of mean indices for individual years were above acceptable levels. The cubic-spline technique has been very useful in some of our analyses of trees from the subarctic and from the closed-canopy forests of the eastern US⁶. This group of trees does not exhibit the coherent, common, low-frequency trends that have been observed in some other subarctic trees²⁻⁴. This could be attributed in part to the standardization method but, additionally, inspection of the cores and the raw data do not show such trends to be as evident as they are in cores from other subarctic sites sampled.

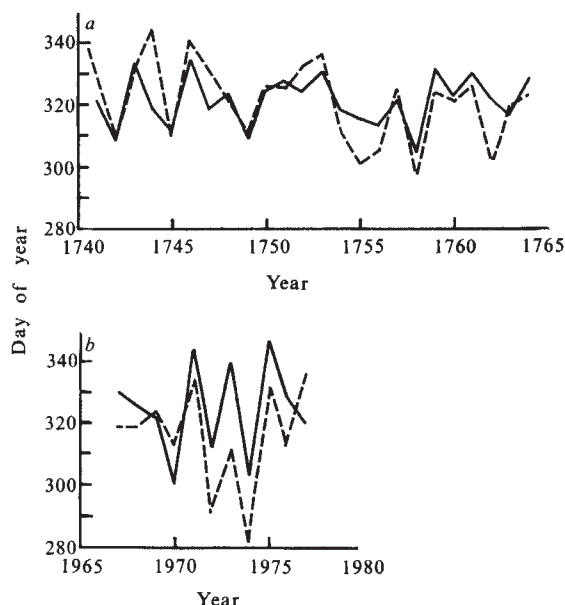


Fig. 2 *a*, Calibration data (dashed line) and reconstructed data (solid line) for first complete freeze of the Churchill Estuary. *b*, Verification data (dashed line) and reconstructed data (solid line) for first complete freeze.

earlier (see Fig. 1 legend). In an attempt to remove much of the nonclimatic variation, the raw ring-width data were standardized using cubic smoothing splines⁶ which removed much of the variance with periods of 100 yr or more in length. The mean sensitivity of the standardized ring-width indices⁷ for this chronology is 0.15, standard deviation (s.d.) is 0.17 and serial correlation is 0.44. Average values for these parameters for subarctic chronologies are 0.16, 0.25 and 0.62 respectively⁴. Based on a common period of 182 yr (1718–1899), the first principal component of the normalized ring-width indices for the cores accounts for 34% of the total variance.

One of the serious problems in reconstructing climate-related phenomena in the subarctic is the shortness of records and the lack of data for verification. The ice record for Churchill is unusual in that there are both recent and historical data. The older records result from an exhaustive analysis of old Hudson Bay Company records by Moodie and Catchpole⁸ who were able to construct time series of quantitative data on ice conditions for several sites near old Hudson Bay Company factories along the south and south-west coast of Hudson Bay.

The historical ice data contain the following parameters for the nearby estuaries: days of first breaking, first partial freezing and first complete freezing. Not all the ice parameters have a clear relationship with climate nor are they equally reliable. The Churchill record is considered most reliable with respect to the records at York Factory, Fort Albany and Moose Factory⁸ (see Fig. 1). The ice parameters at Churchill are, in order of increasing reliability: first partial freezing, first complete freezing and first breaking⁸. A fourth parameter, first ice free, shows very low reliability.

Table 1 Mean and standard deviation of first-full-freeze time series used or reconstructed

	Mean	s.d.
Prince of Wales—all data	321	11.96
Prince of Wales—calibration period (1751–64)	322	11.95
Modern Churchill—all data	317	15.10
Modern Churchill—verification period (1967–77)	317	15.08
Reconstruction (1680–1977)	323	10.31

The first-breaking data are judged to be most reliable from the standpoint of observational accuracy but for the following reasons may be less reliable as a simple climatic parameter. Ice breakup in many north-flowing, high-latitude rivers results from a combination of climatic and hydrological factors. In relatively low-relief regions such as Manitoba, the southern, headwater areas thaw sooner than the northern, outflow areas. The flow of water from the south causes hydraulic pressure and can either overflow or rupture the ice in the more northerly portions of rivers. These hydrodynamic effects of spring meltwater on breakup produce a more complex response to climate than the freeze response.

Modern Churchill River ice data are recorded by the Canadian Meteorological Service and extend from 1957 to 1980⁹. Precise definitions of specific ice events and observer judgement are crucial to the quality of ice record¹. Due to changes in freeze-up definition⁸, an 'approximate only' value for 1965 and a missing value for 1966, we used only the past 14 yr of reliable record for our analyses.

The meteorological data used are from the Churchill A station at the Churchill airport, ~7 km east of the estuary and 1 km north of the tree-core sampling area. This record of mean monthly temperatures and total monthly precipitation starts in January 1943. First subfreezing temperatures occur in September and, in the historical and modern ice records, complete freezing of ice takes place during October or November, usually the latter. Temperature data for these 3 months successfully passed tests for homogeneity^{10,11}.

We concluded that, for this study, any proxy time series should be based on tree-ring samples from the same locality

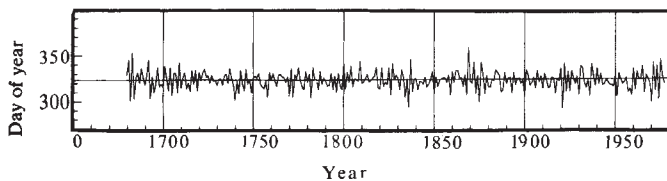


Fig. 3 Day of first complete freeze of the Churchill Estuary, Manitoba, Canada, reconstructed using tree rings, 1680–1978.

as the particular set of ice data because (1) plots of ice-condition time series and tree-ring series from distant locations showed little correspondence; and (2) similarly, the graphical relationships and correlations between the historical ice records at different stations were generally low.

Comparison of graphical plots of tree-ring and ice-condition data from Churchill indicated a correspondence between complete-freeze day and tree growth the next year. Response-function analysis¹² indicated a significant positive relationship between July temperature and ring-width indices as is common in most high-latitude forest-ecotone chronologies⁴. More importantly, a significantly positive ring-width response to warm October and November of the earlier year was shown. Although increased tree growth after a warm autumn the previous year has shown up in other dendroclimatic analyses of subarctic chronologies, generally only one autumn month, November, shows a significant positive relation to radial growth of the next year^{2,3}.

The freezing of the estuary at Churchill may be influenced by river flow and wind effects but, based on correlations with monthly meteorological data, the major apparent influence is autumn temperature. The simple correlation coefficients between complete freeze day and monthly mean air temperatures for September, October and November are 0.67, 0.73 and -0.02, respectively, for the period of reliable modern ice data 1967–79. The first two coefficients are significant at the 95% level, the third obviously not. One can infer that most of the heat loss from the estuarine waters takes place in September and October, although the mean modern full-freeze day is 12 November for this same period.

Two sets of historical ice data comprise the Churchill record, one for Churchill Factory and the other for Fort Prince of Wales (see Fig. 1)⁸. Although the first-complete-freeze subset of data includes more values for Churchill Factory, more of these values have been inferred during the continuous period used for calibration⁸. Therefore, we based our calibration and reconstruction on the Fort Prince of Wales record.

Based on the graphical comparisons, response-function results and the correlations between fall temperatures and freeze-up, we performed multiple linear regression (MLR) using tree growth of the next year and current year as predictors of complete freeze day for the longest continuous freeze-up record, 1741–64. The multiple correlation coefficient (R) is 0.69, variance explained (R^2) is 0.47 and R^2 adjusted for loss of degrees of freedom due to the regression is 0.42 (ref. 13). The standard error of estimate is 9.00 and the F -ratio after the second step is 9.44. The latter is significant beyond the 99% level. The first variable entered is the ring-width index for the following year. This relationship concurs with the response-function result showing a positive relationship between tree growth and a warm autumn the previous year. The negative relationship with current year's growth is due to the difference between autoregressive properties of the tree-ring series and the ice data. The calibration equation resulting from the MLR is

$$\text{Full freeze day} \triangleq 301.7 + 68.5I_{t+1} - 46.6I_t$$

where I_{t+1} = ring width index, following year, and I_t = ring width index, current year.

A regression between Churchill Factory first-complete-freeze data and the tree-ring data is not significantly different from the regression between Fort Prince of Wales record and the tree-ring data for the calibration period (1741–64). MLRs between the other ice parameters from Fort Prince of Wales and Churchill Factory and tree-ring data are not as strong as the first-complete-freeze data results.

Figure 2 compares the reconstructed first-complete-freeze record to the calibration period and the verification period. The entire reconstruction record from 1680 to 1978 is shown in Fig. 3. The reliable portion of the modern record from 1967 onwards, verifies the reconstruction with a correlation coefficient for the common period of 0.62 and a coefficient of determination of 0.38; both are significant at the 95% level. The more rigorous reduction of error statistic (Re) (ref. 14) is +0.14, also verifying some skill at estimating first-full-freeze day. The Re statistic should be viewed with caution in this case as it is unstable for small ($N \leq 20$) data sets¹⁴.

Although 38% explanation of variance is not too impressive, the reconstruction passes the two verification tests. This reconstruction is calibrated by data developed from 200-yr-old descriptive accounts, is based on trees that respond to other factors besides those affecting the full-freeze conditions, and is verified by modern observation. Both the historical and modern ice data involve subjective observer decisions¹ and thus can be sources of error. In view of these difficulties, the graphical comparisons in Fig. 2 and the verification tests are good indications of the validity of the reconstruction.

Temporal and spatial ice variations in the Arctic have an important interactive role with climate¹⁵. Increase and decrease in areal extent of ice responds to climate¹⁶ and also causes feedback into climatic change¹⁷. Very few long-term quantitative records of ice are available. This reconstruction of almost 300 yr demonstrates the feasibility of reconstructing ice data in one location. For this first effort we deliberately chose a favourable estuarine area. Other studies have related tree rings to atmospheric pressure variations¹⁸ and pressure to ice variations^{5,19}. From these earlier studies and the significant relationships indicated by this study, we are confident that additional reconstructions of ice conditions over wider areas can be achieved using tree-ring information.

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Isotope evidence for Pleistocene climatic changes in Kashmir, India

R. V. Krishnamurthy* & Michael J. DeNiro

Department of Earth and Space Sciences, University of California, Los Angeles, California 90024, USA

R. K. Pant

Physical Research Laboratory, Ahmedabad 380 009, India

Stable carbon isotope ratios of organic carbon from palaeosols in loess, and oxygen isotope ratios of pedogenic and lacustrine carbonates reveal significant Pleistocene climatic changes in the Kashmir basin. The extreme $\delta^{13}\text{C}$ values of -16 and -25% reported here indicate a shift from arid to sub-humid conditions. The $\delta^{18}\text{O}$ values of pedogenic carbonates is $\sim 2\%$ higher than that of the underlying lacustrine carbonates, suggesting that they were deposited in different climatic conditions.

The Plio-Pleistocene infillings in the intermontane Kashmir basin preserve a sequence of climatic events in the form of the Karewa sediments which have an estimated thickness of $\sim 1,000$ m and have been identified as glacio-fluvio-lacustrine, capped by a mantle of loess. At various localities in the valley, these sediments have been exposed by tectonic events^{1,2}. Preliminary palaeomagnetic measurements suggest that the upper part of the Karewas are within the Brunhes normal epoch and therefore should preserve a large segment of the Pleistocene climatic record³. Figure 1 shows a schematic section at Burzahom (34°10' N, 74°53' E), ~ 25 km north-east of Srinagar. Member 1 represents a lacustrine regime with CaCO_3 -rich marl layer said to represent periodic drying of the lake. Member 2 is a structureless mud facies representing swampy conditions after the draining out of this lake. Member 3 is a loess deposit of 15 m with three palaeosols and member 4 is the top archaeological deposit. Dirlpur (33°56' N, 74°57' E) section follows the same sequence of events except that member 2 here is a gravelly deposit. Member 3 is ~ 22 m thick while member 4 is absent.

Loess constitutes the top of the Karewas⁴. Loess, a silt-sized sediment, is generally wind-deposited in periglacial environments and represents cold, arid conditions. Palaeosols, or buried soils, in the loess are indicative of warmer/wetter phases. Extensive studies on loess and palaeosols have revealed their value as continental palaeoclimatic indicators⁵⁻⁷.

We have studied the carbon and oxygen isotope variations in the palaeosols and pedogenic carbonates respectively from Burzahom (member 3, Fig. 1). The palaeosols are relatively rich in organic carbon and the pedogenic carbonates occur in dense, nodular forms, deposited through secondary dissolution—precipitation cycles. Oxygen isotope ratios of the lacustrine carbonates from Burzahom (member 1, Fig. 1) pedogenic and lacustrine carbonates from Dirlpur have also been studied.

Buried soils in the loess indicate phases of vegetation growth due to climatic amelioration. The organic fraction of the buried soils is derived largely from the terrestrial plant community which fall into two major categories, C3 and C4, characterized by distinct $\delta^{13}\text{C}$ values and ecological preferences⁸. The C3 plants have an average $\delta^{13}\text{C}$ value of -27% and the C4 plants -14% on the PDB scale. The C4 plants are mostly distributed in deserts and salt marshes. Carbon isotope measurements indicate that while there are differences in isotope ratios among individual metabolites, the total organic matter in a sediment is a reliable indicator of the source ratio⁹. "Most studies of carbon isotopic composition in plants have made use of unfractionated organic matter. Gross differences in metabolism (C3

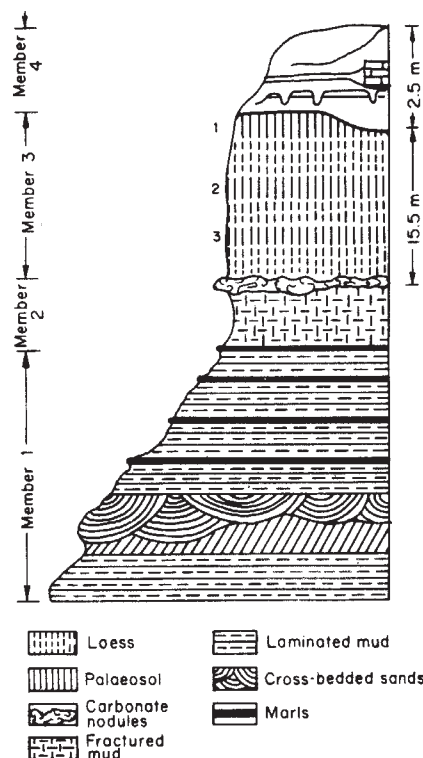


Fig. 1 Schematic section at Burzahom, near Srinagar. Member 1 comprises laminated muds, cross-bedded sands and marls, representing lacustrine and fluvial conditions. Member 2 represents swampy conditions when the lake drained out. Member 3 is the loessic deposit intercalated with three palaeosols (1, 2, 3); the top (member 4) 2.5 m is an archaeological deposit ($\sim 4,500$ yr BP) (not to scale).

compared with C4) can be readily distinguished from such experiments"¹⁰. The $\delta^{13}\text{C}$ values of the organic fraction of a buried soil should therefore document the type of vegetation it supported and hence the ambient climate.

The results of the stable isotope measurements on the organic fraction are shown in Table 1. For organic carbon, samples were first demineralized by 20% HCl, freeze-dried and combusted to give CO_2 using a method described by Stump and Frazer¹¹. Carbonate samples were treated with 'Chlorox' to remove any organics present and reacted with 100% H_3PO_4 at 25 °C. (All the measurements were carried out at the University of California, Los Angeles.)

The $\delta^{13}\text{C}$ values of the organic fractions range from -16.2 to -25% and indicate a clear dominance of the C4 type of plants in palaeosol 2. A gradual increase in the contribution of the C4 type vegetation towards the top of palaeosol 2 indicates an increasing aridity, which merges with the arid, unweathered loessic phase. In this the bottommost layer marks a transition from the laminated member to loess. If the loess deposition there commenced at the termination of a lacustrine regime, therefore, marking the onset of glacial aridity, our isotope measurements point to episodic soil formations through climatic

Table 1 Stable carbon isotope ratios of organic fraction of palaeosols

Site	Depth (cm)	Material	$\delta^{13}\text{C}^*$ (‰)	^{14}C date ($t_{1/2} = 5,568$ yr) (yr BP)
Burzahom	250–300	Palaeosol 1	-25.3	$18,890^{+830}_{-750}$ †
	300–350	Palaeosol 2	-16.2	$>31,000$
	350–400	Palaeosol 2	-18.3	$>31,000$
	400–450	Palaeosol 2	-20.3	
	1,400–1,550	Palaeosol 3	-21.9	$>31,000$

* Reproducibility on a single sample is 0.2%.

† Quoted with 2σ error. The error is one of counting statistics only.

* Permanent address: Physical Research Laboratory, Ahmedabad 380 009, India.

Table 2 Stable oxygen isotope ratios of pedogenic and lacustrine carbonates

Site	Material	$\delta^{18}\text{O}(\text{‰})$	No. of samples analysed
Burzahom	Pedogenic carbonates	-7 ± 0.3	12
	Lacustrine carbonates	-8.8 ± 0.2	15
Dilpur	Pedogenic carbonates	-7 ± 0.3	10
	Lacustrine carbonates	-8.4 ± 0.2	8

* Reproducibility on a single sample is 0.2‰.

amelioration, but not so dramatic as represented by palaeosol 1 when the region had a less arid climate, and a C3 type of vegetation reflected in the $\delta^{13}\text{C}$ value of -25‰ . Note that palaeosol 1 is dated to ~ 18 kyr BP (ref. 12), which represents a relatively more humid climate. The relatively wetter-warmer conditions seem to have continued up to 15 kyr BP at higher altitudes of $\sim 3,000$ m as reflected by the pollen profile at Toshmaidan in Kashmir¹³.

In many areas the period 18 kyr BP coincides with the last glacial maximum¹⁴⁻¹⁷. Peninsular Rajasthan also shows an intense sand dune building at this period¹⁸. This anti-correlation between Kashmir and Rajasthan could be explained either by an early deglaciation in certain parts of the world¹⁹, or better still, by the strengthening of the northeastern monsoon and heavier winter precipitations during this period of weaker southwestern monsoon as indicated by Duplessy²⁰. During the past 3 Myr the rise of the Pir Panjal range ($\approx 5,000$ m) has effectively shielded off the Kashmir valley from the Indian southwestern monsoon (D. P. Agrawal, personal communication). Present-day rainfall pattern in the region is dominated by heavier winter rains as opposed to the southwestern monsoon in most of Peninsular India.

Oxygen isotope ratios of carbonates, precipitated in equilibrium conditions, are determined by the oxygen isotope composition of the water from which they form (ice-volume effect) and the temperature of precipitation (temperature effect)²¹. For lacustrine and pedogenic carbonates, conditions of isotopic equilibrium are generally assumed but not proved. Interpretation of the isotopic record of continental carbonates require also that the 'meteoric effect'²² and possible changes in the local hydrological conditions be taken into account. The meteoric effect makes the carbonates heavier with increasing temperature as opposed to the temperature and ice volume effects, which make the carbonates lighter with increasing temperature. The role played by changing local hydrological conditions are often of an unknown magnitude but could be indirectly climate-controlled. Studies on speleothems have indicated that the meteoric effect may be insignificant thus carbonate isotope record interpretation can be made less complicated²³.

The carbonates that we studied are massive and laminated or large-sized nodules, sandwiched between impervious clay members, and therefore represent sealed environments. Moreover, we have analysed a population of carbonates from various layers to check on possible post-depositional exchanges. The rather negligible scatter within a population suggests minimal such exchanges as any secondary exchange is more likely to produce a wide scatter.

The lacustrine carbonates and the pedogenic carbonates from the loess fall into two oxygen isotope groups, the latter being heavier by 2‰ (Table 2). This depletion in the lacustrine carbonates could point to a warmer period during the lake phase. Sedimentologically, the carbonates are associated with finer particles typical of melt water load, therefore snow melt contribution to the lake, making carbonates lighter, is a distinct possibility. Such an effect also points indirectly to a warmer phase. Therefore, despite the 2‰ variation being small and following the observations by Cerling *et al.*²⁴ and Magaritz *et*

*al.*²⁵, it appears from our preliminary measurements that these sedimentary carbonates may provide useful palaeoclimatic evidence. Analysis of many more carbonates from the basin, together with present day meteoric and lake waters should provide a firmer interpretation of the carbonate isotopic data.

These, the first isotope measurements from the continental sediments of Kashmir, have proved to be useful markers of climatic change. For detailed and definitive palaeoclimatic inferences, much wider data are needed—these are being obtained as part of a 5-yr project.

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An anomalous Suess effect above Europe

A. F. M. de Jong

Vening Meinesz Laboratory, Department of Geochemistry, University of Utrecht, 80.021, 3508 TA Utrecht, The Netherlands

W. G. Mook

Isotope Physics Laboratory, University of Groningen, Westersingel 34, 9718 CM Groningen, The Netherlands

The major impact that man is likely to have on climate comes from the artificial increase of the atmospheric CO_2 level, caused by the combustion of fossil fuels^{1,2}. To predict its magnitude, the future rise of the global atmospheric CO_2 level must be calculated using carbon reservoir models. Because fossil fuel CO_2 lacks ^{14}C and has a $^{13}\text{C}/^{12}\text{C}$ ratio which is 18‰ lower than the present average atmospheric $^{13}\text{C}/^{12}\text{C}$ ratio, the validity of such geochemical models can be tested using past temporal variations in the atmospheric concentration of these isotopes. However, the past variation of the $^{13}\text{C}/^{12}\text{C}$ ratio in global

reservoirs is very difficult to infer³. Using $\Delta^{14}\text{C}$ data of tree rings Suess measured a depletion in the atmospheric ^{14}C concentration over the past century of <10% (ref. 4). Some years later more accurate data gave a value for this 'Suess effect' of 15–25% (ref. 5). As the models have been improved considerably over the past few years^{6–8} the availability of $\Delta^{14}\text{C}$ data with an accuracy of better than 1.5% was thought to be essential to test these^{9,10}. The present results indicate a good correlation with model predictions taking into account natural ^{14}C variations. However, deviations between American and European tree ring data after AD 1920 suggest a European continental CO_2 effect.

As the Netherlands are bordered by the most industrialized regions in Europe, it was suspected that the ^{14}C level of the local air masses was depressed below the average ^{14}C level⁹. We therefore selected two oaks grown in southern Germany near Unterfahlheim (48°24' N, 10°10' E) and Sindelfingen (48°43' N, 9°0' E). Comparison with the absolute master chronology showed that no rings were missing. To avoid the influence of radial transport of material with nuclear-bomb produced ^{14}C , cellulose was extracted from those tree rings which were part of the estimated sap-wood section when the nuclear bomb tests began^{10–12}. All other tree rings were pretreated according to the AAA method¹³. In several cases a combination of material from several tree rings was required

Table 1 Radiocarbon content in tree rings of south German oaks calculated with the half life of 5,730 yr and corrected for isotopic fractionation

Unterfahlheim (48°24' N, 10°10' E)

Year of growth	Laboratory no. GrN-	$\delta^{13}\text{C}_{\text{PDB}}$ (‰)	$\Delta^{14}\text{C}$ (‰)
1948–50	9620*	-25.19	-26.5 ± 1.7
1943–45	9439*	-25.76	-23.8 ± 1.4
1941	9253	-26.70	-24.9 ± 1.6
1937	9290	-26.46	-21.8 ± 1.5
1933	9291	-26.68	-19.6 ± 1.3
1927	9365	-26.99	-17.2 ± 1.1
1925	9386	-26.79	-17.1 ± 1.5
1922	9366	-26.49	-14.1 ± 2.3
1918–21	9440*	-25.37	-15.4 ± 1.4
1917	9367	-26.93	-12.1 ± 1.2

Sindelfingen (48°43' N, 9°0' E)

1931	9387	-25.94	-20.1 ± 0.8
1920	9388	-25.52	-14.4 ± 1.5
1913	9441	-25.52	-11.5 ± 1.4
1909	9442	-25.89	-10.6 ± 1.5
1905–06	9619	-25.30	-10.7 ± 1.1
1901	9477	-25.37	-9.0 ± 1.4
1899–1900	9806	-25.11	-6.0 ± 1.1
1897–98	9621	-25.40	-6.9 ± 1.0
1893	9478	-24.17	-6.7 ± 1.4
1891–92	9805	-25.44	-4.5 ± 1.4
1890	9642	-25.46	-5.0 ± 1.3
1888–89	9773	-25.80	-4.4 ± 1.3
1886–87	9774	-25.15	-5.5 ± 1.3
1884–85	9775	-25.10	-6.8 ± 1.3
1883	9690	-25.57	-6.9 ± 1.4
1881–82	9691	-25.65	-5.8 ± 1.1
1880	9692	-25.61	-5.2 ± 1.0
1878–79	9776	-25.70	-3.7 ± 1.3
1877	9777	-25.00	-5.0 ± 1.3
1875–76	9778	-25.43	-4.4 ± 1.1
1874	9779	-25.65	-8.9 ± 1.1
1872–73	9780	-25.19	-8.2 ± 1.1
1871	9781	-25.30	-4.9 ± 1.2
1869–70	9782	-25.01	-1.8 ± 1.0
1867–68	9783	-25.16	-4.1 ± 1.4
1865–66	9882	-25.09	-4.3 ± 1.2
1863–64	9919	-24.93	-3.9 ± 1.3

* Cellulose pretreatment; the other samples were pretreated according to the standard AAA extraction procedure¹³

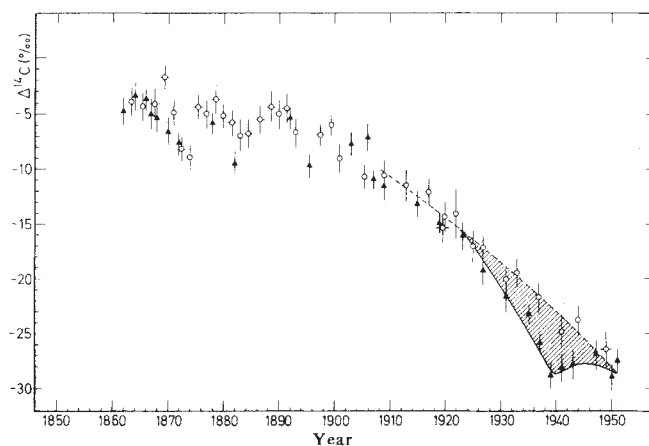


Fig. 1 A compilation of the $\Delta^{14}\text{C}$ results for both the Dutch ($\blacktriangle$) and German ($\circ$) material. The dashed area indicates the local fuel contamination at the Dutch sampling site.

to obtain the 100–200 g of wood necessary for a ^{14}C analysis with a standard deviation of 1.2%. The system stability and counting corrections for the set of seven proportional counters used have been described elsewhere^{12,14}.

The results obtained for the south German oaks are presented in Fig. 1 and are listed in Table 1 as the age ($t_1 = 5,730$ yr) and fractionation corrected deviation from A_{ox} , the standard 0.95% NBS oxalic acid activity:

$$\Delta^{14}\text{C} = \left(\frac{A - A_{\text{ox}}}{A_{\text{ox}}} \right) \times 1,000 \quad (\text{‰})$$

where A_{ox} is corrected to 1950 and -19%, and A to the year of growth and -25%, respectively.

For the period before AD 1900, when the dilution of the atmospheric ^{14}C level was still negligible, our results are in good agreement with the results previously obtained for the Dutch oaks⁹. We suggest that the fine structure superimposed on the rather constant ^{14}C level during the nineteenth century is caused by solar-cycle induced changes in the natural ^{14}C production.

For the period AD 1920–40, however, there is an increasing divergence between the two data sets (Fig. 2). This effect is confirmed by the results obtained for three additional tree ring samples from the Dutch tree (*Quercus rubra*, Sleen) (Fig. 2 and Table 2). The maximum local depression of the atmospheric ^{14}C level at the Dutch sampling site appears to be 26%, with an average of slightly less. This must be caused by a regional contamination. However, after AD 1940 the divergence decreases and disappears around AD 1950. An explanation for this can be found in either a decreased regional effect at the Dutch site or an increased local effect at the German sampling site. To investigate this, our results are compared with ^{14}C data from the US West Coast with a comparable accuracy, covering

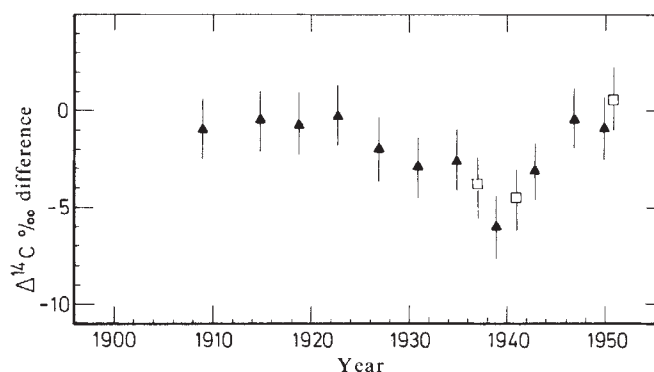


Fig. 2 The calculated difference between the $\Delta^{14}\text{C}$ results for the Dutch oak (*Quercus rubra*, Sleen⁹) and a curve calculated through the ^{14}C results for the German trees. Additional samples ($\square$) confirm the existence of a local fossil fuel effect.

the same time interval. These data were obtained from material from two American Douglass firs¹⁰. These trees grew in oceanic air at the Olympic Peninsula (47°46' N, 124°06' W). Due to a lack of reliable data between AD 1895 and 1915, a comparison could not be made for the entire period (Fig. 3) but three points of interest can be noticed:

(1) During the nineteenth century the average $\Delta^{14}\text{C}$ level between both European and American data sets is almost identical. However, the correlation in fine structure as observed to exist between the Dutch and south German $\Delta^{14}\text{C}$ data sets is absent.

(2) The south German $\Delta^{14}\text{C}$ data also seem to be 2–3‰ lower than the American data during the period AD 1920–40. This would reflect a 10% local depression at the German sampling site.

(3) Also for the German material the divergence seems to decrease after AD 1940. The observation that the Dutch, south German as well as the American data indicate an almost identical ^{14}C level in 1950 must mean that this change can hardly be attributed to a natural variation in the ^{14}C production. This effect would be identical for all three sites and the divergence would be maintained.

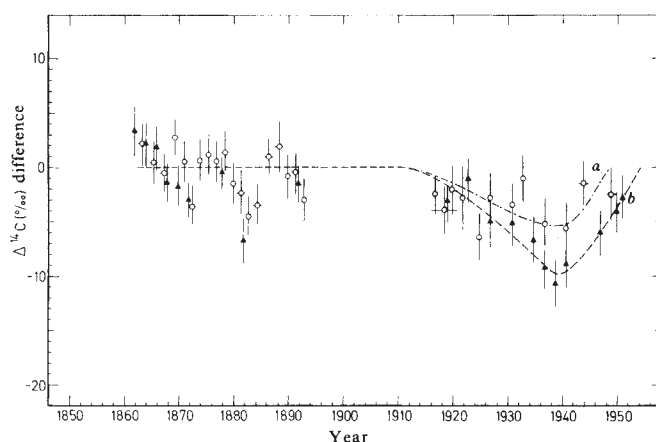


Fig. 3 The calculated difference between the $\Delta^{14}\text{C}$ results for the American Douglass firs and: a, the south German oaks (○); b, the Dutch oak (▲) allowed some curves to be drawn by hand. During the nineteenth century the correlation between $\Delta^{14}\text{C}$ results for the Dutch and German oaks does not exist for the American Douglass fir.

We suggest that this has been caused by changes in the relative rates of local influence. As the American data are believed to be a true representation of the atmospheric ^{14}C concentration above the ocean and therefore more widely representative, the effect most probably indicates that the decrease in fossil fuel consumption during the Second World War was more dramatic in the industrialized part of Western Europe than in the rest of the world.

We conclude that until AD 1920 the time variation in the dilution of the atmospheric ^{14}C level for northwestern and central Europe on the average seems to be identical with the ^{14}C level above the ocean. The results for the period after AD 1920, however, suggest a variable European continental $\Delta^{14}\text{C}$ effect. Large air masses may have a ^{14}C level which differs by 10–30‰ from the ^{14}C level of the oceanic air masses. If this is confirmed by more measurements for material from both the European and American continent, the true global time variation of the atmospheric ^{14}C concentration during the past 150 yr cannot easily be established. If the $\Delta^{14}\text{C}$ data of the Suess effect are obtained from a single tree, this will reduce its use for testing the accuracy of global carbon reservoir models. Only a global sample network, including the Southern Hemisphere, can thus give more information about the size and distribution of the diluted continental air masses in the past.

Although these ^{14}C results are probably only representative of the variations in ^{14}C concentrations of the European air

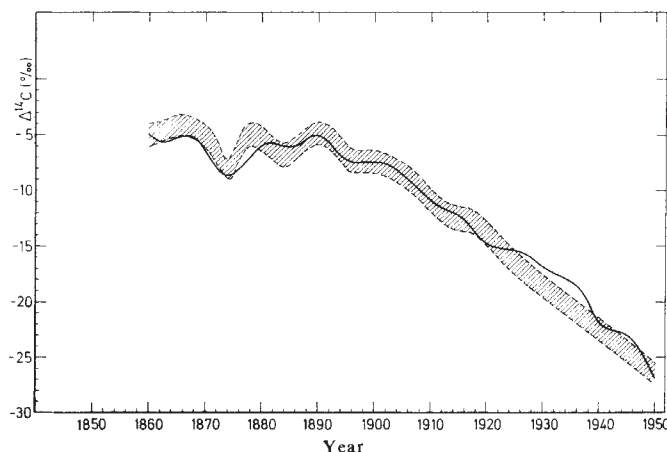


Fig. 4 A comparison of the changes in the atmospheric ^{14}C concentration calculated by model C in ref. 10 (solid curve) with a smoothed spline function with its standard deviation, calculated using the tree ring measurements (dashed area)²⁰. The data from both Dutch and German oaks were used. For the period after AD 1920 the Dutch results were neglected.

masses, we have made a comparison with a recent global reservoir model calculation (Fig. 4)¹⁰ carried out using a box diffusion model^{7,15}. Values of the annual production during the period AD 1860–1950 were taken from ref. 16. In this calculation the varying background ^{14}C level has also been taken into account. Both the long-term geomagnetically- and medium-term solar-induced ^{14}C variations were calculated, respectively, using the relation derived by Houtermans¹⁷ and an empirical relation between the average 11-yr solar cycle sunspot number and the ^{14}C production rate as determined for the pre-industrial interval

Table 2 Radiocarbon content in tree rings of a Dutch oak (*Quercus rubra*, Sleen, 52°45' N, 6°45' E)

Year of growth	Laboratory no. GrN-	$\delta^{13}\text{C}_{\text{PDB}}$ (‰)	$\Delta^{14}\text{C}$ (‰)
1951	9293	-25.45	-27.6 ± 1.2
1941	9480*	-23.90	-28.1 ± 1.3
1937	9479*	-24.65	-25.9 ± 0.9

Radiocarbon content is calculated with the half life 5,730 yr and corrected for isotopic fractionation. More results from this tree have been published previously⁹.

* Cellulose pretreatment; the other sample was pretreated according to the standard AAA extraction procedure¹³.

AD 1723–1885¹⁸. A short-term annual variation has been introduced superimposed on the medium-term contribution, as established from neutron flux observations^{18,19}.

If we now compare our ^{14}C results with these model calculations then apart from the local dilution effect after AD 1920, the fine structure during the nineteenth century is in rather good agreement. Because during that period there was only a minor effect of fossil fuel combustion, the influence on the variation in the model-calculated ^{14}C level is minimal. Therefore, the influence of changes in the solar activity on the ^{14}C production within an 11-yr solar cycle, as given in the model, seems to explain quite acceptably our measured short-term ^{14}C variations. However, especially for this fine structure, which can hardly be influenced by regional effects during this period, a better correlation between trees grown at different sites in the Northern Hemisphere is thought to be essential to establish a ^{14}C variation modulated by the 11-yr solar cycle.

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Triassic bedded cherts in central Japan are not pelagic

Ryuichi Sugisaki, Koshi Yamamoto & Mamoru Adachi

Department of Earth Sciences, Nagoya University, Nagoya, Japan

Although the origin of bedded radiolarian cherts in orogenic belts has been widely debated^{1–15}, their geochemistry is still poorly understood. Triassic bedded radiolarian cherts occur extensively in the Japanese Islands and are generally not associated with ophiolitic rocks but rather with clastic rocks. We present here chemical data on the Triassic bedded cherts of Kamiasso in central Japan. The relationship of SiO₂ with Al₂O₃, TiO₂ and Zr shows that the bedded cherts have been formed by a simple mixing of biogenic silica derived from radiolarians and sponge spicules with lithogenic materials. The ratios of several elements relative to TiO₂ strongly suggest that the shale partings and lithogenic portions of the cherts are con-sanguineous. Sedimentary structures as well as the geochemical evidence suggest that most Kamiasso cherts are turbidity current deposits. Our geochemical and geological data compared with those of recent marine sediments from various environments, demonstrate that the Triassic cherts and associated shale partings are not deep pelagic in origin but formed in an offshore or marginal sea environment.

Samples were collected from an extensive continuous outcrop of a Triassic chert formation^{16–18} along the Hida river, Kamiasso, Gifu Prefecture, central Japan (Fig. 1). The chert formation, ~100 m thick, is conformably overlain by a series of Lower(?)–Middle Jurassic strata¹⁹ of chert, siliceous shale and turbidite and is underlain by Triassic siliceous shale with chert layers. No ophiolitic rocks occur spatially associated with the cherts. The bedded chert consists of rhythmically interbedded chert and thinner shale (Fig. 1a); chert layers are from 1 to 30 cm thick, mostly 2–7 cm, and shale layers from paper-thin to 1.5 cm thick. The chert has a wide colour range: green, grey, dark red-brown, red, white and black. Some dark red-brown chert has a less vitreous appearance and yields best-preserved Triassic radiolarians. Bedding surfaces are generally smooth, but where shale partings become thicker they tend to be knobby and pinch-and-swell structures and load casts are common. The chert layers occasionally show parallel lamination, whereas graded bedding is rare and inconspicuous because detrital grains of quartz, feldspar or lithic fragments are not present in the cherts. Locally, graded bedding is displayed either by the upwards increase of clay content or by the upwards size grading of siliceous remains within one lamina. The chert is made up largely of cryptocrystalline quartz with a small amount of illite, chlorite and opaques; radiolarian tests and sponge spicules filled with cryptocrystalline quartz or length-fast chalcedony are ubiquitous (Fig. 1b). Some radiolarians are cut by vertical microstylolites. Secondary minerals such as quartz, chlorite, carbonate and opaques are generally present in trace amounts except for white chert which is invariably veined by microcrystalline quartz and chalcedony.

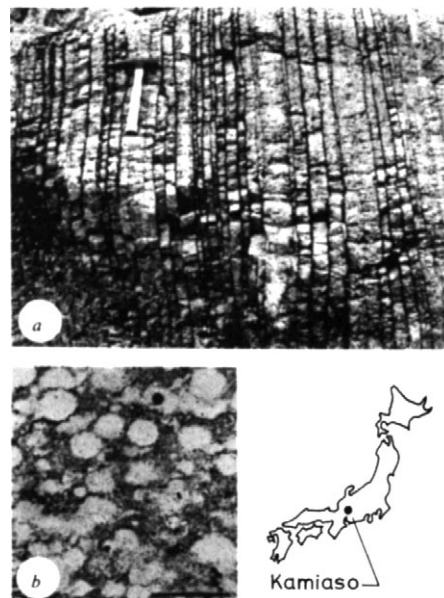


Fig. 1 Triassic bedded radiolarian chert at Kamiasso, Gifu Prefecture, central Japan. *a*, Rhythmically interbedded chert and thinner shale (hammer head, 20 cm); *b*, photomicrograph of black chert containing abundant silica-filled tests of radiolaria (scale bar, 0.2 mm).

The shale partings are very fissile and soft compared with cherts, have the same colour as associated cherts, and are composed essentially of a fine aggregate of illite, quartz, chlorite and opaques with or without radiolarian remains. Some partings have a conspicuous fracture cleavage with flattened radiolarians and well-oriented clay minerals.

Sixty-nine chert samples and 37 shale partings collected from the outcrop were analysed with an automatic X-ray fluorescence spectrometer (Table 1). Figure 2 shows the SiO₂ content in cherts and shale partings plotted against Al₂O₃, TiO₂ and Zr. That the composition of the chert samples trends to SiO₂ = 100% indicates that pure silica from a separate source is diluting other components during sedimentation. We thus conclude that the SiO₂ of the bedded cherts can be regarded as biogenic; the biogenic silica in each sample experienced different degrees of mixing with other elements which are considered mostly as lithogenic.

To examine the depositional environment of the Triassic cherts, the analytical data were compared with those of about 750 marine argillaceous sediments, which were collected during

Table 1 Chemical composition of cherts and shale partings in averages and their standard deviations (water, carbonate free basis)

	Cherts	Shale partings
No. of analyses	69	37
(%)		
SiO ₂	96.22 ± 2.23	63.60 ± 6.48
TiO ₂	0.077 ± 0.04	0.77 ± 0.16
Al ₂ O ₃	1.74 ± 0.86	16.98 ± 3.11
Total Fe ₂ O ₃	0.77 ± 0.38	6.37 ± 1.16
MnO	0.018 ± 0.012	0.13 ± 0.15
MgO	0.44 ± 0.30	2.48 ± 0.51
CaO	0.35 ± 0.02	0.19 ± 0.22
Na ₂ O	0.082 ± 0.023	0.17 ± 0.24
K ₂ O	0.40 ± 0.23	4.81 ± 0.95
P ₂ O ₅	0.035 ± 0.015	0.24 ± 0.16
(p.p.m.)		
Co	3.3 ± 2.2	17 ± 11
Ni	14 ± 5.4	66 ± 22
Cu	60 ± 26	126 ± 101
Zr	23 ± 23	164 ± 47
Pb	8.2 ± 6.0	29 ± 34

Table 2 TiO₂ normalized values of marine sediments* in various regions and Triassic cherts and shale partings in central Japan

Sediment type	Area	Environment	Cruise (ref.)	Distance from land (km)	Water depth (m)	No. of samples analysed	Al ₂ O ₃ /TiO ₂	Total Fe ₂ O ₃ /TiO ₂	MnO/TiO ₂
Marine argillaceous sediments	Nishitsugaru (140° E, 41° N)	Continental shelf and slope	GH77-3II (25)	30	1,400	87	24.11 ± 1.98	8.73 ± 0.75	0.109 ± 0.112
Marine argillaceous sediments	Pacific Coast, South-west Japan	Continental shelf and slope, Nankai Trough	GH75-4 (20)	110	2,900	29	23.11 ± 1.99	8.35 ± 0.51	0.121 ± 0.078
Marine argillaceous sediments	Pacific Coast, North-east Japan	Continental shelf and slope, Japan Trench	GH76-2 (26)	150	3,800	67	22.16 ± 1.20	9.23 ± 1.14	0.112 ± 0.044
Marine argillaceous sediments	Central Sea of Japan	Marginal sea	GH78-2 (21)	260	2,300	61	22.72 ± 1.59	9.30 ± 1.64	0.298 ± 0.596
Marine argillaceous sediments	Shikoku Basin	Marginal sea	Site 443 (22)	480	4,400	114	23.54 ± 1.51	9.08 ± 0.72	0.488 ± 0.221
Marine argillaceous sediments	Shikoku Basin	Marginal sea	Site 444 (22)	560	4,800	74	22.82 ± 0.56	9.16 ± 0.45	0.455 ± 0.296
Marine argillaceous sediments†	Around Ogasawara Ridge	Around basaltic islands	GH79-2, 3, 4 (23)	930	2,600	32	18.52 ± 1.76	11.09 ± 0.95	0.295 ± 0.117
Marine argillaceous sediments‡	Ogasawara Trench	Trench	GH79-2, 3, 4 (23)	600	7,400	40	21.90 ± 0.86	10.40 ± 0.50	0.569 ± 0.508
Pelagic sediments	Pacific Ocean floor on the east of the Ogasawara Trench	Deep ocean floor	GH79-2, 3, 4 (23)	820	5,600	22	21.98 ± 1.04	10.28 ± 0.40	0.971 ± 0.720
Pelagic sediments	Northern Central Pacific Basin	Deep ocean floor	GH79-1 (24)	1,000<	5,200	34	19.63 ± 3.41	9.82 ± 0.80	0.497 ± 0.289
Pelagic sediments	Northern Central Pacific Basin, Wake to Tahiti	Deep ocean floor	GH80-1 (27)	1,000<	5,500	72	23.28 ± 2.05	11.83 ± 0.91	2.11 ± 0.60
Pelagic sediments§	Northern Central Pacific Basin, Wake to Tahiti	On the basaltic plateau	GH80-1 (27)	1,000<	4,700	15	12.36 ± 2.30	8.40 ± 1.25	1.33 ± 0.40
Pelagic sediments	Northern Central Pacific Basin (174° W, 10° N)	Deep ocean floor	GH80-4 (28)	1,000<	6,000	76	22.97 ± 1.66	10.95 ± 1.21	2.19 ± 0.80
Marine argillaceous sediments	Northern Central Pacific Basin (174° W, 10° N)	Deep ocean floor	GH80-4 (28)	1,000<	6,000	31	27.82 ± 2.73	13.09 ± 0.96	3.52 ± 0.85
Radiolarian cherts	Kamiaso, central Japan	'Chichibu geo-syncline'				69	22.08 ± 2.32	10.21 ± 2.44	0.254 ± 0.196
Shale partings	Kamiaso, central Japan	'Chichibu geo-syncline'				37	21.97 ± 1.07	8.89 ± 2.03	0.168 ± 0.191

* Samples of marine sediments are mostly collected from piston cores and deep sea drilling cores. 'Distance from land' and 'water depth' are expressed as values in averaged locations of a core group.

† The low Al₂O₃/TiO₂ ratio can be attributed to basalts of the Ogasawara Islands.

‡ Some samples of this group show high ratios of Al₂O₃/TiO₂ and high content of SiO₂ (ref. 23); these contain much acidic tuff.

§ The low Al₂O₃/TiO₂ ratio can be attributed to basalts from the Manihiki Plateau.

|| The high Al₂O₃/TiO₂ ratio is due to zeolites which are derived from acidic tuff.

cruises of the Geological Survey of Japan and Deep Sea Drilling Project (DSDP) and analysed in our laboratory with the same method used for cherts²⁰⁻²⁸. Si, Al, Ti and Zr tend to remain in the resistates and hydrozates during the sedimentation process: accordingly, the mutual abundances of these elements generally remain stationary during weathering and transportation. We examined the relative abundances of these 'conservative' elements by using TiO₂ concentration as a standard. For example, the Al₂O₃/TiO₂ ratio of sediment hardly changes, even if the sediment is diluted by biogenic silica. Thus, several oxides were normalized to TiO₂ for the marine argillaceous sediments in several regions (Table 2, Fig. 3). We noticed that the ratios of Al₂O₃/TiO₂ and total Fe₂O₃/TiO₂ fall in a narrow range in each region with a few exceptions; the ratios are from 22 to 24 and from 8 to 11, respectively. It is particularly interesting that the ratios are similar to mean values of those of granites and basalts (Fig. 3) because this suggests that the lithogenic part of the marine argillaceous sediments is derived mostly from the continental crust. The exceptions can be attributed to the contribution from oceanic island basalt, acidic tuff and others as noted in Table 2.

The chemical features of the shale partings in the bedded cherts can be compared with those of marine argillaceous sediments to examine the environment of formation for the cherts. The average ratios of Al₂O₃/TiO₂ and total Fe₂O₃/TiO₂ fall into the general range for the marine argillaceous sediments (Table 2, Fig. 3). The partings, however, are especially lacking in CaO and Na₂O (Table 1). This feature is usually observed in fault clay and gouge²⁹, suggesting that elements other than the 'conservative' elements would be soluble through weathering. As the shale partings are very fissile compared with cherts, meteoric waters will selectively soak into the partings and wash out mobile elements, as is the case with shear zones of faults. Accordingly, the elements other than the 'conservative' elements in the shale are of no use for comparison.

On the other hand, the average ratio of Al₂O₃/TiO₂ in the cherts (22.1) is remarkably close to that of the shale partings (22.0) in spite of errors in sampling and analysis. The ratios of total Fe₂O₃/TiO₂ in the chert and in the shale are also similar to each other, although Fe cannot be regarded as conservative. This similarity strongly suggests that the shale partings and lithogenic portions of the cherts are consanguineous. This is

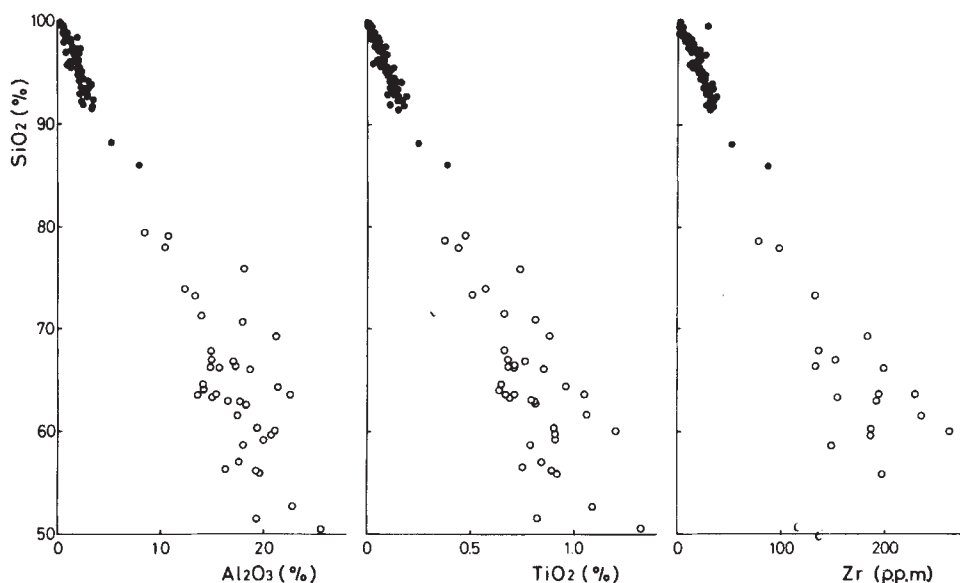


Fig. 2 Relation between SiO_2 and Al_2O_3 , TiO_2 and Zr. ●, Cherts; ○, shale partings.

also implicitly shown in Fig. 2 where points for cherts and those for partings are approximately aligned. Cherts, which resist weathering, are expected to have kept their original composition: most TiO_2 normalized compositions, except for $\text{SiO}_2/\text{TiO}_2$ in the cherts, therefore, show the chemical feature of the lithogenic portion deposited with biogenic silica. From this, we can deduce the sedimentation environment of the lithogenic portion and ultimately that of the whole chert.

Most cherts are the same colour as associated shales; some cherts display parallel lamination and/or graded bedding, and some appear to grade upwards into overlying shales. These data strongly suggest that the Triassic bedded cherts of Kamiasso are turbidites^{2,6,7,9,14}, derived mostly from a previously gathered accumulation of biogenic silica, shale partings being deposited as the tail of the currents. This conclusion is compatible with the chemical inference that the shale partings are consanguineous to the lithogenic portions of cherts.

Support for a turbidite origin of Kamiasso cherts also comes from microfossil evidence: both late Triassic conodonts and early Jurassic radiolarians occur together in some green cherts of the uppermost part of the chert formation¹⁸. A similar reworking of conodonts is also known in the bedded cherts of Inuyama³⁰, ~20 km south of Kamiasso, which are stratigraphically almost in the same horizon as of Kamiasso. Presumably the conodonts would have been reworked at times when the original silica-rich material was transported from shallow sites to deeper depositional basins by turbidity currents. Because detrital grains other than fine clayey materials are not contained in both the cherts and shales, the depositional sites must have been sea basins where little or no coarser terrigenous sediments reached. Where did such basins occur during Triassic time, and where can we find such basins in the modern ocean?

A useful criterion for discriminating depositional environments of marine sediments is the MnO/TiO_2 ratio. This ratio in nearshore sediments remains generally low and does not fluctuate much because granites and basalts regarded as a main source of the sediments have a uniform and low ratio (Fig. 3). As the distance from land and the water depth increase, terrigenous materials decrease while Mn precipitates nearly uniformly from ocean water (Table 2, Fig. 3). The ratio shows almost two orders of magnitude of variation from the nearshore area to the abyssal area. The MnO/TiO_2 ratio of the Triassic cherts under consideration is 0.254 ± 0.196 and is close to those of the argillaceous sediments from the Central Sea of Japan and the continental shelf and slope off South-west Japan. The distance from land to both areas is at most 300 km. The fact that the ratio of the Triassic bedded cherts is quite different from that of deep ocean sediments implies that the cherts are not deep pelagic but would have formed in an offshore or shallow marginal sea within ~300 km from land. Furthermore,

our preliminary analyses of 37 cherts from DSDP Leg 32 give 6.28 for the average MnO/TiO_2 ratio, which is much larger than that of the Triassic cherts. The study of rare-earth elements on DSDP and on-land cherts suggests that some Triassic radiolarian cherts of Kamiasso formed in coastal areas, marginal seas or land-enclosed seas¹¹. All these facts confirm our view that the Triassic bedded cherts in central Japan are not pelagic in origin.

Another factor enhancing the MnO/TiO_2 ratio is the contribution of hydrothermal components resulting from submarine volcanism³¹. For example, Jurassic cherts overlying ophiolites

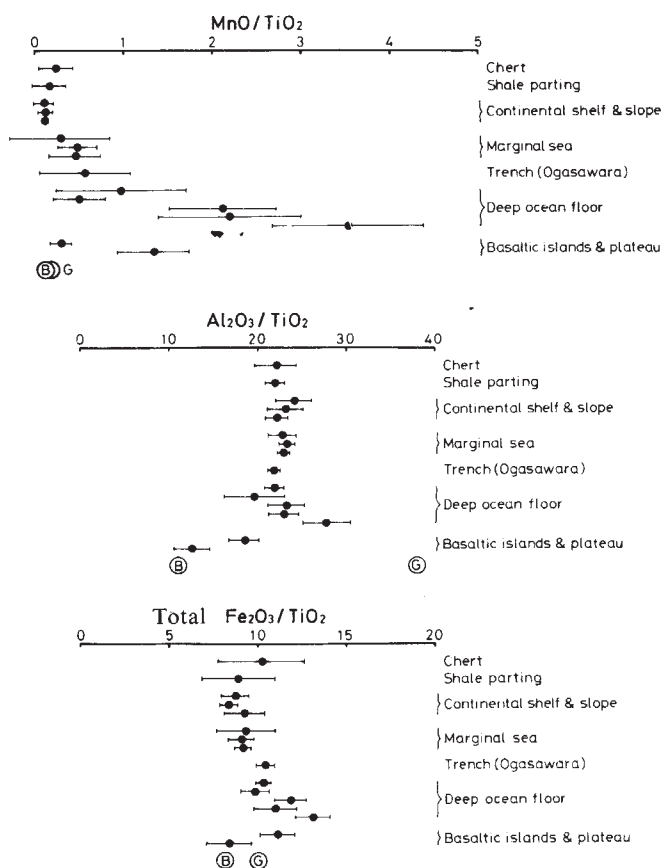


Fig. 3 Comparison of TiO_2 normalized values of marine argillaceous sediments in various environments and Triassic cherts and shale partings. The detailed description is listed in Table 2. The horizontal line indicates $\pm 1\sigma$. G and B represent the averaged values of granites and basalts, respectively³⁴.

in Italy show a higher range of the ratio (0.53–1.40) (refs 4, 32) and they contain much Ni, Co and Cu. The features of these elemental abundance and Pb isotopic compositions³³ have been ascribed to hydrothermal activity during submarine volcanism which gave rise to ophiolites. The MnO/TiO₂ ratio and the concentration of Co, Ni and Cu in the Japanese Triassic bedded cherts (Tables 1, 2) are significantly lower than those in the Italian cherts. This suggests that bedded cherts in close

association with ophiolites as in Steinmann Trinity are different in genesis from those intercalated within sedimentary sequences treated here. Clearly the study of bedded cherts from geochemical aspects in various orogenic belts will provide an important key to understanding the environment of ancient geosynclines.

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Mid-Palaeozoic travels of Arctic-Alaska

J. F. Sweeney*

Earth Physics Branch, Department of Energy, Mines and Resources, Ottawa, Canada K1A 0Y3

One of the most persistent proposals¹ for the origin of the Arctic sea floor is that Alaska, or parts thereof, broke away from polar Canada and rotated as much as 70° anticlockwise relative to the North American craton. Subsequent modifications^{2–8} reduced the size of the rotated block (Fig. 1) and put the date of rotation at latest Jurassic–early Cretaceous (refs 4, 6, 7, 9, 10, and A. Grantz and S. May in preparation). Although restoration of the block provides a good geological fit between northern Alaska and the Arctic islands for late Palaeozoic and younger pre-rift age rocks,⁸ the character of Devonian and older Palaeozoic rocks across the restored boundary is anomalous, with deformed and thermally altered rocks of northern Alaska lying against unaltered sediments on Banks Island (Fig. 2b). Here I compare the known geology of mid- and early Palaeozoic rocks along the margins of the restored rift and explain the anomalous Arctic-Alaska terrane as a block that was displaced southwestwards in mid-Palaeozoic time by as much as 2,000 km from its initial orogenic site to the north and east of Ellesmere Island.

Devonian and older rocks outcrop in northern Greenland¹¹, northern Axel Heiberg and Ellesmere islands¹² and northern Alaska¹³ and are found in the subsurface along the modern polar margin on Ellef Ringnes and Brock islands¹⁴, Banks Island¹⁵, beneath the Mackenzie Delta¹⁶, in northernmost Alaska, Chukotsk Peninsula and the adjacent continental shelf¹⁷. Across northern Greenland and adjacent parts of Canada, shallow-dipping Cambrian platform rocks pass northward from the craton into dominantly carbonate shelf facies which in turn grade northward into a dominantly clastic filled ensialic trough that on Ellesmere Island was two-sided and bounded on the north by a volcanic arc^{11,12,18}. Trough sediments on Ellesmere Island were derived from both margins although the source to the north declined steadily during the Cambrian interval¹⁸. At this time in northern Greenland, detritus was derived from cratonic sources to the south¹⁹.

In early and middle Ordovician time, felsic to intermediate volcanism prevailed on northernmost Ellesmere Island and was followed by local deformation and rapid deposition of flysch derived, at least in part, from Cambrian or older low-grade metamorphic rocks^{12,18} within a major new or rejuvenated source terrane to the north (Fig. 2c). This terrane also delivered clastic debris to northern Greenland beginning in Ordovician time¹⁹. Clastic deposition advanced southward in both regions during the Silurian and early Devonian periods^{12,18,19} and on Ellesmere Island both the volume and grade of deposited material increased to the north-east¹⁸.

To the west of Mackenzie Delta across northeastern Alaska outcrop and borehole data indicate that in Cambrian and Ordovician time shallow-water carbonate arches graded northward into shale–chert basinal sequences that contained substantial volcanic deposits^{13,16,17,20}. In later Ordovician and Silurian time this setting was interrupted by major uplift (Fig. 2c) within the shale–chert zone and was accompanied by deformation, emplacement of a felsic pluton at 431 ± 13 Myr (K/Ar hornblende date)^{20,21} and erosion that exposed Cambrian and older low-grade metamorphic assemblages along the uplift axis¹³. The grain size of early and (?) middle Devonian clastic debris in northernmost Alaska increased towards what is now the west along the present coast¹³.

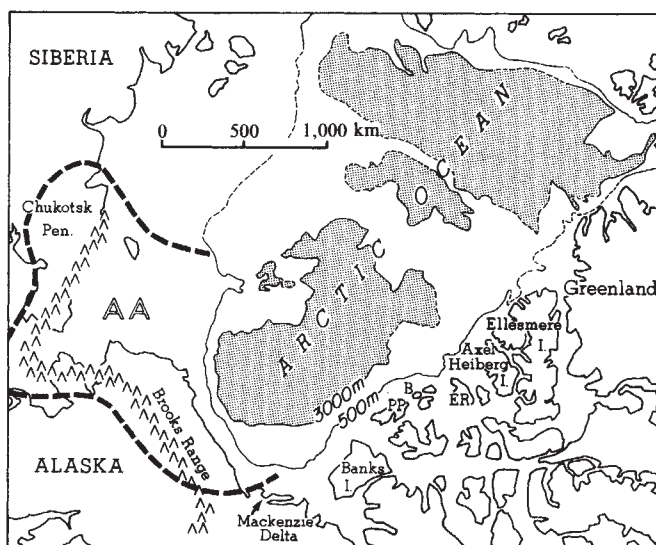


Fig. 1 Boundaries of the terrane^{26,31} designated Arctic-Alaska⁶ (AA), presumed to have rotated. B, Brock Island; ER, Ellef Ringnes Island; PP, Prince Patrick Island.

* Present address: Pacific Geoscience Centre, PO Box 6000, Sidney, British Columbia, Canada V8L 4B2.

Plutonic rocks occur mainly along the southern Brooks Range in a relatively narrow belt (Fig. 2a) that extends northeastwards into the Mackenzie Delta region²¹. A second belt of small intrusions is found along northernmost Axel Heiberg and Ellesmere islands¹². Intrusive rocks in each belt are dominantly felsic and most were emplaced during middle and late Devonian time^{12,21}. Several small ultramafic to felsic bodies of late Silurian or early Devonian age are present within the Axel Heiberg–Ellesmere belt^{12,18}. In both regions, shallow marine conditions at the time of emplacement suggest that the plutonic rocks may represent an ensialic island arc or a continental margin magmatic belt^{18,21}.

Metamorphic grade declines westward from amphibolite facies in northeastern Greenland¹¹ to greenschist–subgreenschist facies with local amphibolites on northernmost Ellesmere Island^{18,22} to virtually unmetamorphosed rocks at Ellef Ringnes Island and beyond¹⁴ (Fig. 2a). West from the Mackenzie Delta thermal effects are variable and reach greenschist grade along the coast but amphibolite grade rocks are associated with the magmatic arc in the southern Brooks Range^{17,21,23}.

Except for the Banks Island area, these regions experienced one or more early Palaeozoic periods of thermal and structural alteration that generally increased in intensity towards the north and culminated during middle Devonian to early Mississippian

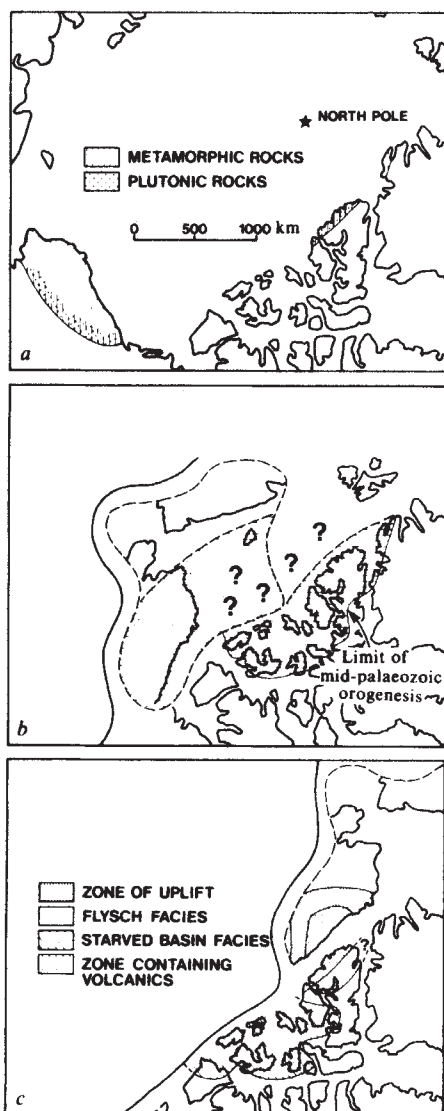


Fig. 2 Early and mid-Palaeozoic orogenic features^{12,13,17,18,21} with continental blocks arranged: a, as at present; b, as they may have appeared before presumed rotation of Arctic-Alaska; c, before onset of mid-Palaeozoic tectonism.

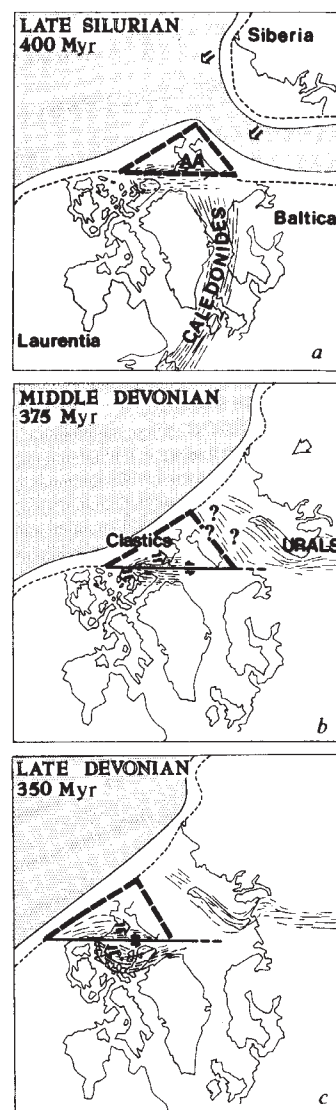


Fig. 3 Possible Devonian tectonic development of the Arctic region. Large arrows in b and c indicate expected directions of sediment and tectonic transport on opposite sides of the fault zone. a, Late Silurian 400 Myr; b, middle Devonian 375 Myr; c, late Devonian 350 Myr.

time with the deposition of thick southward-tapering clastic sediments that palaeocurrent studies suggest were transported mainly from north-east to south-west in both northern Alaska and the Canadian Arctic Islands^{12,13,24,25}. Uplift and clastic wedge formation that began on northern Ellesmere and Axel Heiberg islands in early Middle Devonian time expanded south-westwards at an average rate close to 3 cm yr^{-1} to include Prince Patrick Island by the outset of the Mississippian²⁴. A similar orogenic progression is not documented in northern Alaska where, as in Arctic Canada, the mid-Palaeozoic zone of uplift and related clastic deposition extends hundreds of kilometres south of the present continental margin^{13,25} (Fig. 2b).

In the early 1970s it was proposed that from Greenland to Alaska, the major source of early and mid-Palaeozoic sediments may have been offshore orogenic welts produced by subduction of sea floor beneath the Laurentian continental mass, followed in mid-Palaeozoic time by closure of an ancestral Arctic Ocean and suturing of the opposing cratonic blocks^{2,11,12}. The Banks Island area in this view lay opposite an embayment in the approaching landmass and was therefore spared the orogenic effects of continent–continent collision¹⁵. In a restored rotation, however, no appropriate embayment is evident in palaeogeographical reconstructions of northern Alaska¹⁶. More recent studies indicate that, in Canada, early and mid-Palaeozoic clas-

tic sources to the north did not extend further south-west than Axel Heiberg Island^{18,24} and also that significant left-lateral movements may have been associated with development of the orogen.

The gross similarity in mid- and early Palaeozoic stratigraphy, tectonism and metamorphic grade between Arctic-Alaska and northeastern Laurentia has long been noted^{12,17}. Several of these comparative features may be explained if the two terranes were once joined, polar margin to polar margin (Figs 2c, 3a). The ensialic trough and associated volcanism noted for the Cambrian in the northern part of each region may indicate the presence of an adjacent consuming margin that ceased activity when the opposing Arctic-Alaska block collided with northeastern Laurentia in middle Ordovician time^{18,19}. Uplift, metamorphism, deformation and plutonism in northern Alaska at about this time is matched by contemporary metamorphism, deformation and volcanism on northernmost Ellesmere Island. Uplift in Arctic-Alaska may thereby have become the source region for Ordovician through early Devonian clastics derived from the north in the Greenland-Ellesmere region (Fig. 2c). The crystalline fraction of detritus delivered to the latter region and rocks within the core of the eroded zone in Alaska are similar in age and metamorphic grade. Further, with Arctic-Alaska so oriented, the present westerly coarsening of early Devonian sediments in coastal Alaska becomes, in mid-Palaeozoic time, a northeasterly coarsening in agreement with the textural trend noted in contemporary clastics on what was then the adjacent landmass of Ellesmere Island. For middle Devonian to early Mississippian syntectonic deposits, however, the proposed reconstruction places major sediment transport directions within adjacent terranes in opposition, northeasterly in Arctic-Alaska, southwesterly in Arctic Canada²⁶.

Preliminary palaeomagnetic reconstructions^{27,28} show that the Siberian Shield may have approached the ancestral polar margin of Laurentia from what is now the east during the early Palaeozoic and that intervening sea floor was probably eliminated during Devonian time (Fig. 3a, b). The approach of the Siberian block may have ruptured the weld between Arctic-Alaska and northeastern Laurentia and caused the former to move with compression accompanied by significant left-lateral motion against the Laurentian front to produce a broad zone of uplift and erosion within each block. In Laurentia, the affected zone expanded to the south-west as the Arctic-Alaska block proceeded (Fig. 3b, c). Significant but undated south-west-directed thrusting is also documented on northernmost Ellesmere Island¹⁸. By early Mississippian time, the migrating Arctic-Alaska terrane presumably came to rest against a zone that included the Banks Island area although, as earlier noted, the deformed belt did not extend beyond Prince Patrick Island. Final late- to post-orogenic movements in the region may not have been attended by significant disruption along the zone of contact between the two plates.

This reconstruction implies that Arctic-Alaska also moved against oceanic lithosphere as it advanced to the south-west (Fig. 3). Underthrusting or subduction of the latter beneath the encroaching continental block in middle and late Devonian time may be indicated by the belt of plutonic rocks along the southern Brooks Range (Fig. 2a), which would have been close to the leading edge of the Arctic-Alaska block during this interval (Fig. 3b, c). Contemporary magmatism in northernmost Ellesmere Island may be related to the initial stages of this event. Late Silurian-early Devonian intrusions within the Ellesmere belt may mark the line of separation between Laurentia and Arctic-Alaska or they could be the intrusive equivalents of nearby Ordovician volcanics¹⁸ (Fig. 2c).

A key feature of this concept is that compression combined with left-lateral translation between adjacent continental blocks produces a region of uplift and clastic deposition that advances in each terrane as the zone of contact between them progresses. In Laurentia, the contact zone is seen to move to the south-west as does the region of middle and late Devonian uplift and clastic deposition²⁴ (Fig. 3b, c). In Arctic-Alaska, the opposing

Laurentian block will appear to move to the north-east. Mid-Palaeozoic uplift and sediment transport in Arctic-Alaska should therefore proceed also towards the north-east. That is, the relative motions should produce overall directions of orogenic expansion and sediment transport in each block that, when compared, will be diametrically opposed. As indicated earlier, this is the case for mid-Palaeozoic palaeocurrent directions when Arctic-Alaska is placed in its pre-rotated position against polar Canada^{25,26}. However, there is little evidence of corresponding northeastward orogenic expansion at this time in northern Alaska, perhaps because the entire facing side of the smaller Arctic-Alaska block was already in contact with the larger Laurentian block at the start of the tectonic episode (Fig. 3a).

This scheme was anticipated as early as 1965 by Harland²⁹ who proposed left-lateral shearing north of Greenland and Ellesmere Island as an extension of major sinistral faulting in late Devonian time within the Caledonian foldbelt. Recent palaeomagnetic evidence³⁰ assigns a Carboniferous age to the latter displacement which implies that Devonian shearing along the polar margin was an earlier and therefore separate event as suggested here.

The present scenario is attractive in that it explains early Palaeozoic and Devonian geological and tectonic similarities between what are now widely separated regions and, in a restored rotation, it accounts for dissimilarities between pre-Carboniferous sequences in the Banks Island area and in northern Alaska. The location as well as the linearity of the present polar margin from Greenland to the Mackenzie Delta are also explained as having been the site of a major mid-Palaeozoic shear.

At the end of Jurassic time, some 200 Myr after its Palaeozoic travels came to an end, Arctic-Alaska moved again, this time in anticlockwise rotation to begin the process of opening the modern Arctic Ocean basin.

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A mutation in *Drosophila* alters normal connectivity between two identified neurones

John B. Thomas & Robert J. Wyman

Department of Biology, Yale University, Box 6666, New Haven, Connecticut 06511, USA

The mechanisms involved in producing the vast number of specific connections in the nervous system are unknown. As nerve cells grow during development and seek out their targets there must be some sort of molecular cues for axonal guidance and final target recognition. One strategy for identifying such factors is to locate the genes which code for or control the expression of the molecules. To identify these genes, we have chosen a simple network of eight neurones in *Drosophila melanogaster*, comprising the giant fibre (GF) system¹⁻³, in which we can detect the effect of mutations on connectivity. We report here the isolation of an X-linked, recessive mutation, *bendless* (*ben*), which deletes one process of the GF while leaving other portions functionally unaltered.

The GF network is activated by a light-off visual stimulus and mediates the early events of an escape response consisting of a jump and subsequent flight⁴. To isolate potential connectivity mutants we used standard mutagenesis techniques and a behavioural screen to select flies failing to jump to the light-off stimulus. Males 1 or 2 days old were fed the mutagen ethyl methane sulphonate (EMS)⁵ and mated to *C(1)M3/Y* females⁶; the F₁ male progeny from this cross inherit the mutagenized paternal X chromosome. We screened 50,000 F₁ males. The screening device consists of a flask inverted in a beaker. Flies are placed inside the flask and are free to walk along its inner surface. A light-off stimulus causes them to jump, but because activation of the flight muscle does not always result in maintained flight, the flies fall a small distance. After several stimuli, jumpers fall into the beaker where they are unable to re-enter the testing area; non-jumpers remain in the flask. White-eyed flies, which lack the visual screening pigments, show greater reliability than wild type in jumping to the light-off stimulus. Therefore, white-eyed flies of the *brown*; *scarlet* (*bw*; *st*) genotype were used. Both the morphology of the GF and its motor output in *bw*; *st* flies are indistinguishable from wild type.

Non-jumping lines were established from individuals selected in the screen. Males from these mutant lines were then tested physiologically for defects in the GF system by stimulating the GFs extracellularly in the brain and recording from thoracic muscles known to be driven by them³. Underlying structural abnormalities in the GF were revealed by intracellularly injecting the GF with the fluorescent dye Lucifer yellow. From these experiments several X-linked mutations have been isolated which disrupt the normal physiology and morphology of GF system neurones. The *bendless* mutation is one of these and maps between *vermillion* and *forked*.

The GFs themselves are a pair of large interneurons having cell bodies located ipsilaterally in the brain². Each sends an axon along the dorsal midline of the cervical connective to the thoracic ganglion where it courses ventrally. Within the mesothoracic neuromere the GF axon makes a lateral bend and ends abruptly^{1,2} (Fig. 1*a, b*). Just before this lateral bend, the GF makes an electrical synapse with the peripherally synapsing interneurone (PSI)¹, which crosses the ganglion and chemically synapses onto the five motoneurons of the contralateral dorsal longitudinal flight muscle (DLM). At the terminus of its bend, the GF makes another electrical synapse with a

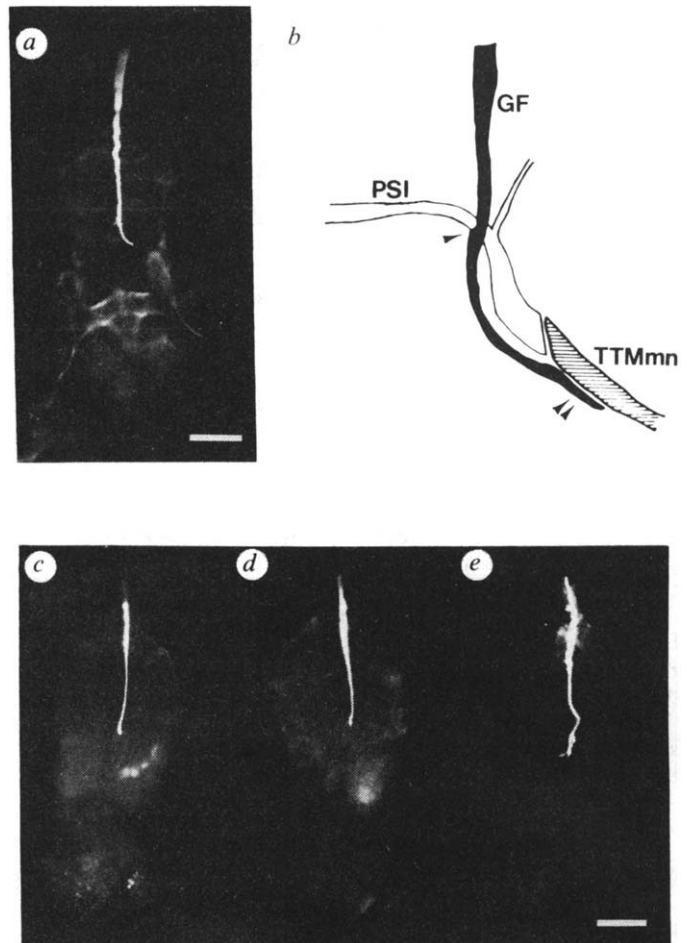


Fig. 1 *a, c-e*, Whole mounted thoracic ganglia, viewed with fluorescence optics, from a wild-type (*a*) and three *bendless* (*c-e*) individuals. In each the GF has been injected with Lucifer yellow. Techniques of dye injection are as described by Koto *et al.*². *a, c, d*, Dorsal views; *e*, a dorso-lateral view in which the normal ventral bend and abnormal branches are visible. Anterior is up; scale bar, 100 μm. *b*, Schematic diagram of the GF bend region from an electron micrograph reconstruction¹ showing the location of synapses between the GF and PSI (single arrowhead) and between the GF and TTM motoneurone (double arrowhead).

large motoneurone innervating the tergotrochanteral muscle (TTM, or jump muscle)¹.

In mutant flies (*n* = 10) the GF cell body position and dendritic morphology are indistinguishable from wild type (data not shown) and the GF axon descends unbranched to the thoracic ganglion. However, within the ganglion the GF fails to make the lateral bend characteristic of wild type and terminates before making the bend (Fig. 1*c, d*). Extending from the tip are numerous fine processes ~3–5 μm long. Occasionally, this branching is more extensive (*n* = 2), consisting of processes up to 30 μm long (Fig. 1*e*). In neither case does the GF extend to the region where it normally makes synaptic contact with the TTM motoneurone. The TTM motoneurone itself is present and drives the TTM normally. Preliminary experiments using retrograde labelling with horseradish peroxidase indicate that the TTM motoneurone morphology is normal in *ben* flies (M. Koto, personal communication).

Physiological experiments show that in the mutant the GF drives the DLM normally, but the TTM is driven abnormally. In wild type a spike in the GF drives the TTM and DLM with short and constant latencies. Figure 2*a* shows the response recorded in the TTM and DLM to intracellular stimulation of the GF in a wild-type individual. The TTM, whose motoneurone is driven monosynaptically by the GF, has a response latency

of 0.8 ms; the DLM, whose motoneurons are driven disynaptically via the PSI, has a response latency of 1.2 ms. Figure 2b shows the GF output to the TTM and DLM of a *ben* individual. It can be seen that the DLM response latency of the mutant is indistinguishable from that of wild type. However, the TTM latency is abnormally long at 2.2 ms. All *ben* flies examined physiologically ($n = 40$) had abnormal TTM response latencies of 2.2–3.0 ms. Whether the giant fibre in the mutant actually does make synaptic contact with the TTM motoneurone, albeit in an abnormal region, is not known. However, as the TTM latency is 1.4–2.2 ms greater than wild type, and a normal synaptic delay is of the order of 0.5 ms, it is unlikely that there is direct functional contact between the GF and the TTM motoneurone. Instead, it is likely that the GF is driving the TTM via an as yet unidentified polysynaptic pathway.

Evidence from following frequency experiments supports the conclusion that the normal GF–TTM motoneurone synapse is absent. The TTM of wild-type flies can be driven by GF stimulation at frequencies >100 Hz (ref. 3), but in the mutant, the TTM can be driven 1:1 only at frequencies <1 Hz. In contrast, the normal DLM following frequency to GF stimulation is unaffected in the mutant. Figure 3a shows the response in the TTM of wild type to extracellular stimulation of the GF at 50 Hz. The pathway can respond to each of the stimuli with its characteristically short and constant latency. Figure 3b shows the response in the TTM of a *ben* individual to GF stimulation at 50 Hz. The pathway can only follow the first stimulus. As the DLM responded normally to all stimuli in the train, each stimulus was effective in eliciting a spike in the GF. Figure 3c shows that the TTM pathway of *ben* flies is capable of following

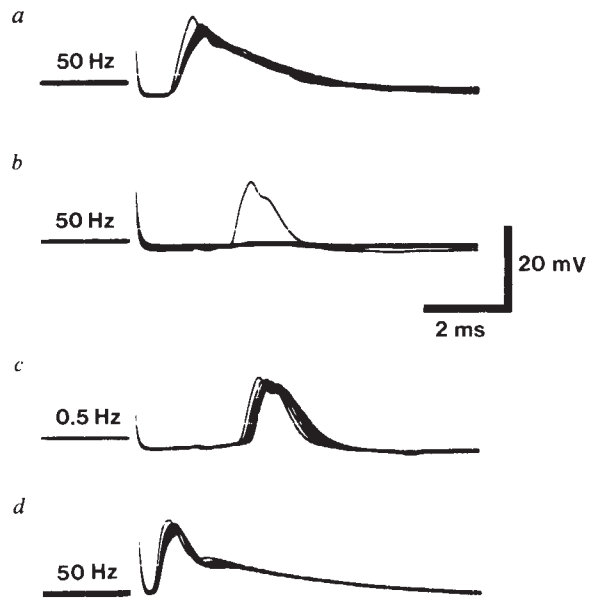


Fig. 3 *a*, GF stimulation at 50 Hz while recording the response in the TTM of a wild-type individual. *b*, Same as *a* but in the mutant *ben*. *c*, GF stimulation at 0.5 Hz while recording the response in the TTM of a *ben* individual. *d*, TTM motoneurone stimulation at 50 Hz in *ben*. In each trace 10 effective stimuli were delivered. Insulated tungsten electrodes were used for stimulating and recording in all experiments.

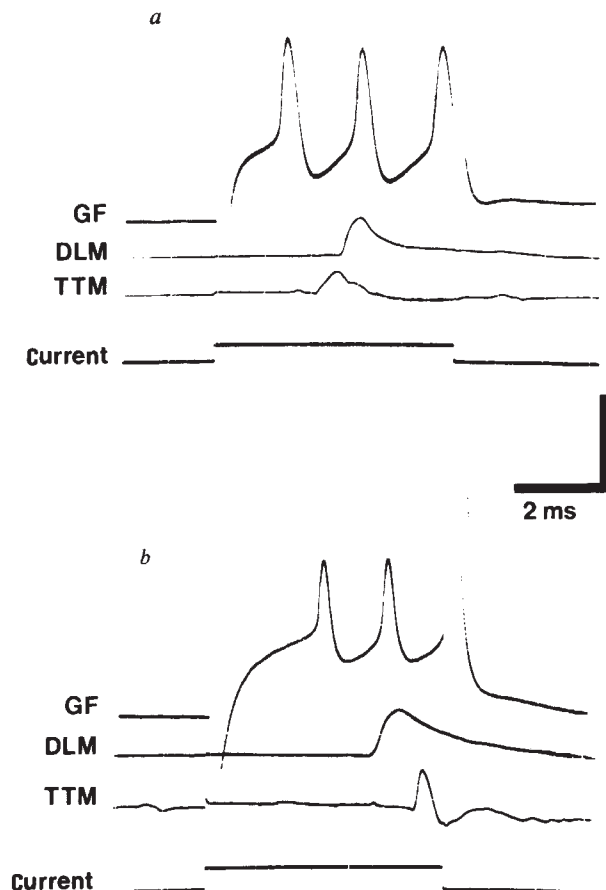


Fig. 2 Response in TTM and DLM to intracellular stimulation of the GF in wild-type (*a*) and *ben* (*b*). Top traces are the intracellular records of the GF. Current is shown in the lower traces. Muscle responses shown in the middle trace are driven by the first GF spike. Vertical calibration is 40 mV for the GF and DLM, 20 mV for the TTM; 10 nA for the current.

low frequencies of GF stimulation without failure. In this experiment the GF was stimulated at a frequency of 0.5 Hz; the TTM followed 1:1. To exclude the possibility of a defect in motoneurone spike conduction, neuromuscular transmission or the muscle membrane, the TTM motoneurone was stimulated extracellularly in the ganglion while recording from the TTM itself. Figure 3d shows the TTM response to stimulation of its motoneurone at 50 Hz; the muscle follows the train of stimuli without a failure. This indicates that the site of lability in the pathway mediating the abnormal TTM response lies centrally, between the GF and TTM motoneurone.

One of the more interesting possibilities for the mutation's mechanism of action is that some molecule necessary for the recognition between the GF and the TTM motoneurone is either missing or defective in the mutant. During normal development, insect neurones often send out many branches and retract them at approximately the time synaptic contact is made with their targets⁷. In this case, the presence of abnormal branches in *ben* may be the result of the GF failing to make normal synaptic contact with the TTM motoneurone. Further genetic analysis of mutations such as *ben* may lead to the identification of genes encoding developmentally important molecules involved in connectivity. Ultimately, the molecules themselves may be identified using the techniques of molecular biology.

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Cross-species neural grafting in a rat model of Parkinson's disease

Anders Björklund, Ulf Stenevi, Stephen B. Dunnett & Fred H. Gage

Department of Histology, University of Lund, Lund, Sweden

Previous studies¹⁻⁷ have shown that intracerebral implants of embryonic mesencephalic dopamine (DA) neurones, grafted between individuals of the same inbred rat strain, can reverse some of the functional deficits caused by damage to the nigrostriatal DA pathway. These observations have raised the possibility that the intracerebral neural grafting technique may eventually find a clinical application in the treatment of neurodegenerative disorders, particularly Parkinson's disease. One obvious obstacle to any such attempts is the immunological rejection mechanisms associated with allogeneic or xenogeneic grafting. Thus, neural tissue (sensory and sympathetic ganglia), transplanted across immunological barriers to sites outside the central nervous system (CNS), are known to be rejected^{8,9}. The brain has, however, been described as an immunologically 'privileged' site, partly perhaps because of its protective blood-brain barrier¹⁰⁻¹². This may be the case for grafts of embryonic tissue, in particular. Thus, Zalewski *et al.*¹³ have reported rejection of allografts of adult ganglionic neurones transplanted to the spinal cord, while Low *et al.*¹⁴ found prolonged survival of embryonic brain tissue in the hippocampal region, grafted between rats of different strains. We report here long-lasting functional cross-species transplantation of mesencephalic DA neurones, taken from mouse embryos, to the dopaminergically denervated neostriatum of adult recipient rats.

Grafts were taken from the ventral mesencephalon (containing the developing substantia nigra-ventral tegmental area DA cell group) of 16-17-day-old mouse embryos (NMRI-strain, Anticimex, Stockholm). The recipients were 18 young adult female rats (Sprague-Dawley strain, Anticimex AB) subjected to a unilateral 6-hydroxydopamine (6-OHDA) lesion of the right nigrostriatal DA pathway 1 month before transplantation. The lesion was performed by local injection of 8 µg 6-OHDA-HCl (Hässle) into the nigrostriatal pathway¹⁵. The lesion of the nigrostriatal DA pathway was complete in all recipients as assessed by the intensity of the amphetamine-induced turning response. The criterion rate >6 full ipsilateral turns per min over 90 min in response to 5 mg per kg metamphphetamine (intraperitoneally) has previously been shown to correspond to a permanent reduction of neostriatal DA of more than 97% (refs 3, 16). The rotation tests were made in automated rotometer bowls according to Ungerstedt and Arbuthnott¹⁷. The grafts were placed on the dorsal surface of the denervated neostriatum, within a cavity made by aspiration through the anterior parietal cortex and the corpus callosum, as described elsewhere^{1,3}. Further rotation tests were conducted 1½, 3½ and 5½ months after transplantation. Turning in response to a low dose of apomorphine (0.05 mg per kg, subcutaneously) was tested twice, at 4 and 5 months after transplantation. All animals were killed at 6 months after transplantation and the brains processed for catecholamine fluorescence histochemistry according to the ALFA method¹⁸. Alternate sections were stained with cresyl violet.

In 10 of the 18 rats the fluorescence histochemistry revealed surviving DA neurones and reinnervation of the denervated neostriatum. In the remaining 8 animals the transplantation cavities were empty (except for some connective tissue and residual Gelfoam remnants) and there was no trace of surviving DA neurones, suggesting that the grafts in these cases had been

totally resorbed (Fig. 1b). In the 10 positive cases the actual transplant piece, within the cavity, had also been largely resorbed. The surviving DA-containing neurones occurred either in small, apparently non-vascularized cell clusters at the surface of the caudate-putamen, or scattered within the dorsal part of the caudate-putamen (Fig. 1a). These DA neurones were mostly small and rounded, and exhibited a DA fluorescence of widely varying intensity. The cresyl violet stain suggested the presence also of some non-DA neurones in the cell clusters, but there were no obvious signs of lymphocytic infiltration within these clusters, or within the caudate-putamen of the host.

The most striking microscopic finding was the newly formed DA-fluorescent terminal network within the caudate-putamen of the positive cases. In 8 of the 10 specimens this reinnervation was rich and covered large areas of the head of the host caudate-putamen (Fig. 1a). In several cases the DA fibres occurred in patches which were seemingly derived from single DA-containing cell bodies in their vicinity. In two of the positive cases the ingrowth of DA fibres was poor and confined to a few small patches close to the transplantation cavity.

For behavioural analysis the rats were allocated in a blind manner to one of three groups: rich ingrowth, poor ingrowth or no ingrowth. The amphetamine and apomorphine rotation tests revealed a striking relationship between graft ingrowth and compensation of the turning response in the lesioned rats (Fig. 2). Thus, whereas the rats with no DA neurone survival (open circles in Fig. 2a) increased their amphetamine-induced turning response, from an average of 8.1 turns per min before grafting, to an average of 15 turns per min by 5½ months, the rats with DA neurone survival and rich ingrowth into the caudate-putamen (filled circles in Fig. 2a) reduced their turning response from 9.6 turns per min to 1.0 turns per min. The compensation of the turning response developed over the first 3½ months and remained stable between 3½ and 5½ months after transplantation. Both the magnitude and the time course of compensation of the turning response in the xenografted animals are very similar to those previously observed in rats with homologous nigral grafts³. In the rats with poor DA ingrowth into the caudate-putamen, the compensation of the amphetamine-induced turning followed a different time course and became evident only between 3½ and 5½ months after transplantation (open triangles in Fig. 2a). The differences between the groups are statistically significant (Fig. 2).

The contralateral turning response to a low dose of the DA receptor agonist, apomorphine (0.05 mg per kg), which can be taken as a measure of the degree of DA receptor supersensitivity in the denervated neostriatum¹⁹, was on the average reduced by 65-75% in the richly reinnervated rats compared with the rats with no or poor DA ingrowth (Fig. 2b). This reduction is statistically significant and similar to that previously recorded from rats with homologous grafts⁵.

The present results demonstrate that long-term survival of cross-species grafts of embryonic DA neurones can be achieved in the adult rat CNS in the absence of any immunosuppressive treatment. It is interesting that the surviving DA neurones were found on the surface and in the depth of the host caudate-putamen, whereas the bulk of the discrete transplant had been almost totally resorbed. This suggests that the migratory capacity of the embryonic neurones or neuroblasts in the graft is of critical importance for their ability to escape rejection. Although a blood-brain barrier is known to develop within grafts of embryonic CNS tissue, it is likely that it is established by vessels derived from the graft itself rather than from the host^{20,21}. Thus, if the blood-brain barrier is important for long-term survival of immunologically incompatible grafts in the CNS, our results would support the idea that grafted neurones can escape rejection by migration into the protected environment behind the barrier of the host. This would also explain the ability of the xenogeneic DA neurones to reinnervate and establish a new, long-lasting terminal network within the host caudate-putamen.

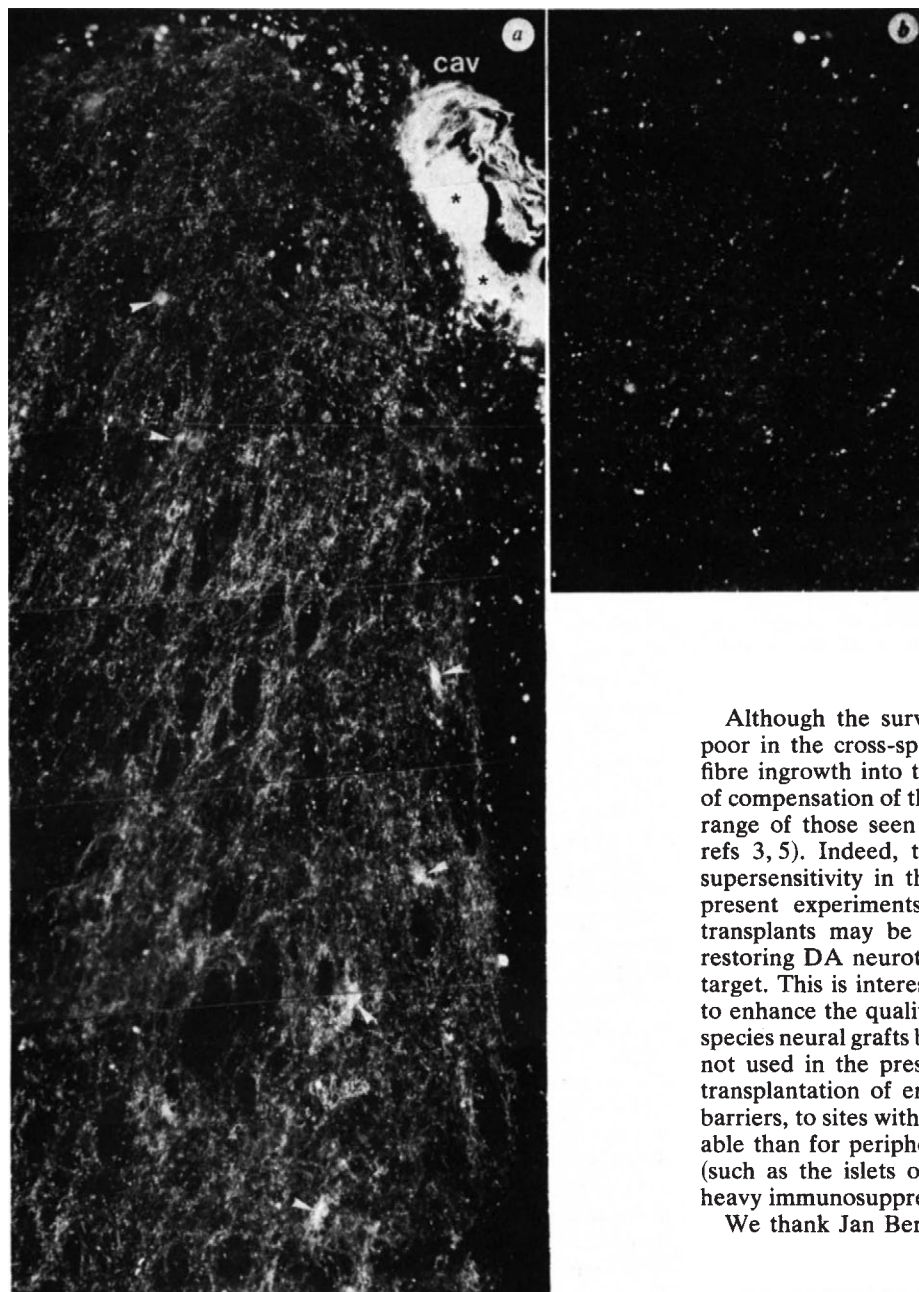
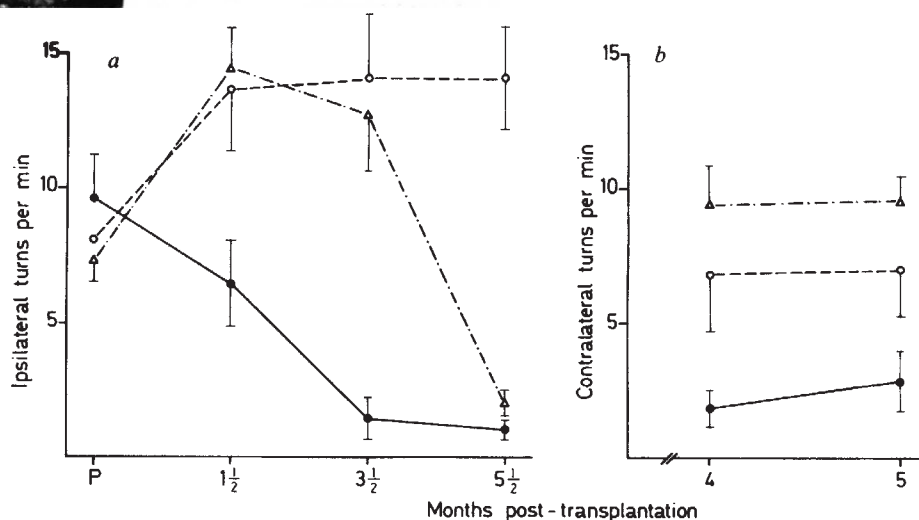


Fig. 1 The dorsal part of the caudate-putamen: *a*, in a case with surviving grafted DA neurones and a rich dopaminergic reinnervation of the dorsal caudate-putamen, and *b*, in a case with no surviving graft and a persisting total dopaminergic denervation of the caudate-putamen. Both rats were killed 6 months after transplantation. Asterisks in *a* denote clusters of intensely fluorescent DA-containing neurones at the bottom of the transplantation cavity (cav). Some fluorescent DA-containing cells within the caudate-putamen of the host are marked with arrow heads. ($\times 160$.)

Although the survival of the discrete grafts was generally poor in the cross-species experiments, the magnitude of DA fibre ingrowth into the host caudate-putamen and the degree of compensation of the turning asymmetry were well within the range of those seen with homologous nigral grafts (compare refs 3, 5). Indeed, the degree of reduction in DA receptor supersensitivity in the reinnervated neostriatum, seen in the present experiments, suggests that the cross-species nigral transplants may be as effective as the homotypic grafts in restoring DA neurotransmission in the previously denervated target. This is interesting not least because it may be possible to enhance the quality and magnitude of survival of the cross-species neural grafts by applying immunosuppressive treatment, not used in the present study. The conditions for functional transplantation of embryonic neurones across immunological barriers, to sites within the brain, thus seem to be more favourable than for peripheral grafts of neurones or endocrine cells (such as the islets of Langerhans) which are rejected unless heavy immunosuppressive treatments are applied^{8,9,22-24}.

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Fig. 2 Drug-induced rotation in rats with unilateral 6-OHDA lesions of the nigrostriatal pathway, followed 1 month later by grafts of DA-rich embryonic mouse mesencephalon to the neostriatum on the same side as the initial lesion. Rats were allocated to three groups on the basis of subsequent blind histological analysis: $\circ$ — $\circ$, no detectable surviving fluorescent DA cells or fibre ingrowth ($n=8$); Δ — Δ , poor fibre ingrowth in small patches in the host rat caudate-putamen ($n=2$); $\bullet$ — $\bullet$, rich surviving fluorescent fibre ingrowth in dorsal portions of the host caudate-putamen ($n=8$). Values plotted are means $\pm$ s.e.m. *a*, Ipsilateral rotation over 90 min following intraperitoneal injection of 5.0 mg per kg metamphetamine, before (*P*) and at regular intervals following transplantation. Difference between groups: $F(2, 15) = 10.59$, $P < 0.01$; group $\times$ time interaction: $F(6, 45) = 9.41$, $P < 0.01$. *b*, Contralateral rotation over 40 min following subcutaneous (s.c.) injection of 0.05 mg per kg apomorphine, 4 and 5 months following transplantation. Difference between groups: $F(2, 15) = 3.97$, $P < 0.05$; group $\times$ time interaction: $F(6, 45) = 0.23$, not significant.



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Association amacrine cells could mediate directional selectivity in pigeon retina

Andrew P. Mariani

Laboratory of Vision Research, National Eye Institute, National Institutes of Health, Bethesda, Maryland 20205, USA

Ganglion cell receptive fields in the vertebrate retina are classified as 'simple' if they display mutually antagonistic, concentric on and off responses to stimulation by light¹, or 'complex' if they respond maximally to a stimulus which moves in a certain direction or has a particular orientation². One complex unit frequently reported is directionally selective^{3–6}. It is excited by stimuli moving through its receptive field in only one direction, the preferred direction, and is inhibited by motion in the reverse or null direction. In species such as cats and monkeys which have a predominantly simple centre-surround organization of ganglion cell receptive fields, there is a low ratio of conventional (amacrine cell) to ribbon (bipolar cell) synapses in the inner plexiform layer, while in species such as pigeons and frogs which have a large percentage of complex ganglion cell receptive fields, there is a high ratio of conventional to ribbon synapses. It is therefore thought that amacrine cells, which are laterally interconnecting neurones in the inner plexiform layer, form the complex ganglion cell receptive field^{7,8}. Although quantitative electron microscopy of the inner plexiform layer has indicated that amacrine cells may form the complex receptive field properties of ganglion cells, it offers no clues to the particular morphology of amacrine cells involved. I have therefore now studied amacrine cells in pigeon retina using Golgi impregnation in both whole-mounted and radially sectioned material to see whether there is a correlation between the morphology of amacrine cells and the receptive fields of ganglion cells. In 30 Golgi-impregnated retinas, I have found many different morphological types of amacrine cell. One of these is so clearly different from all other amacrine cells that it requires the separation of pigeon amacrine cells into two distinct classes, those which lack axons (class I cells) and those which have axons (class II cells).

Class I amacrine cells have dendrites radially organized with respect to their cell bodies when viewed in flat preparations (Fig. 1*b–e*). The cells have diverse morphology, and may include cells with dendritic trees which are multiply stratified, but which nevertheless display a radial symmetry when viewed in flat preparations. In comparison class II amacrine cells of the pigeon retina are not radially organized. In addition to their dendritic processes, they have a short axon with a terminal arborization

in the same stratum of the inner plexiform layer as their dendrites. These cells were first described by Cajal⁹ in another avian species, and he termed them 'association' amacrine cells, but their presence has not been reported since that time¹⁰. They differ from 'ordinary' amacrine cells (class I cells) as they clearly have an axon.

In the 30 Golgi preparations of the pigeon retina, the association amacrine cells are frequently stained: over a 100 may be found in a single retina, but only 5 have been stained completely through their axon terminal arborization.

Association amacrine cells (Fig. 1*a*) have 13 μm diameter cell bodies, which makes them larger than all other amacrine cells (6–10 μm), and are situated in the innermost part of the inner nuclear layer where they are exceeded in size only by the Dogiel (displaced ganglion) cells. Four or five dendrites about 2 μm thick arise from the vitreal part of the cell body and branch once or twice. Large 3–4 μm spiny varicosities occur on the dendrites near branch points, and the dendrites then taper to very fine processes and terminate in the outermost stratum of the inner plexiform layer, with a total length of no more than 35 μm . An axon of 1.5 μm diameter arises directly from the cell body or one of the dendrites and courses just beneath the inner nuclear layer for 200–700 μm . Collaterals may arise from the axon, but two axons are rarely observed; if the two axons do exist, both follow the same course. At its terminal arborization in the outermost stratum of the inner plexiform layer the axon thins to 0.5 μm , bears many 1–2 μm terminal and preterminal boutons and spans up to 200 μm . The axons of different cells appear to be non-preferentially oriented: those arising from nearby cells may cross each other at various angles. The association amacrine cells, due to their morphological polarization, might be involved in the organization of directionally selective receptive fields of ganglion cells in pigeon retina.

Directionally selective units in the pigeon retina respond to movement through their receptive fields in the preferred direction with a vigorous discharge of spikes^{4,5}. There is virtually no response to a moving stimulus in the opposite or null direction³ and a small response to a stationary stimulus over the receptive field⁴. The null directional movements, which are inhibitory and determine the null-preferred axes, are oriented in many directions so that different directionally selective units may have different null-preferred axes⁶. Other evidence³ indicates that there is an inhibitory patch offset from the receptive field centre which determines the null direction, and when examined at this offset patch, the receptive field is non-directional as it is inhibited by moving stimuli in both the null-preferred and orthogonal axes. Similarly, the field centre is non-directional when examined at the preferred side since it may be activated by preferred, null and orthogonal movements³.

Association amacrine cells could be the anatomical basis for the directionally selective responses recorded in the pigeon retina. Their having an axon which separates morphologically distinct parts of the cell is clearly consistent with a physiological

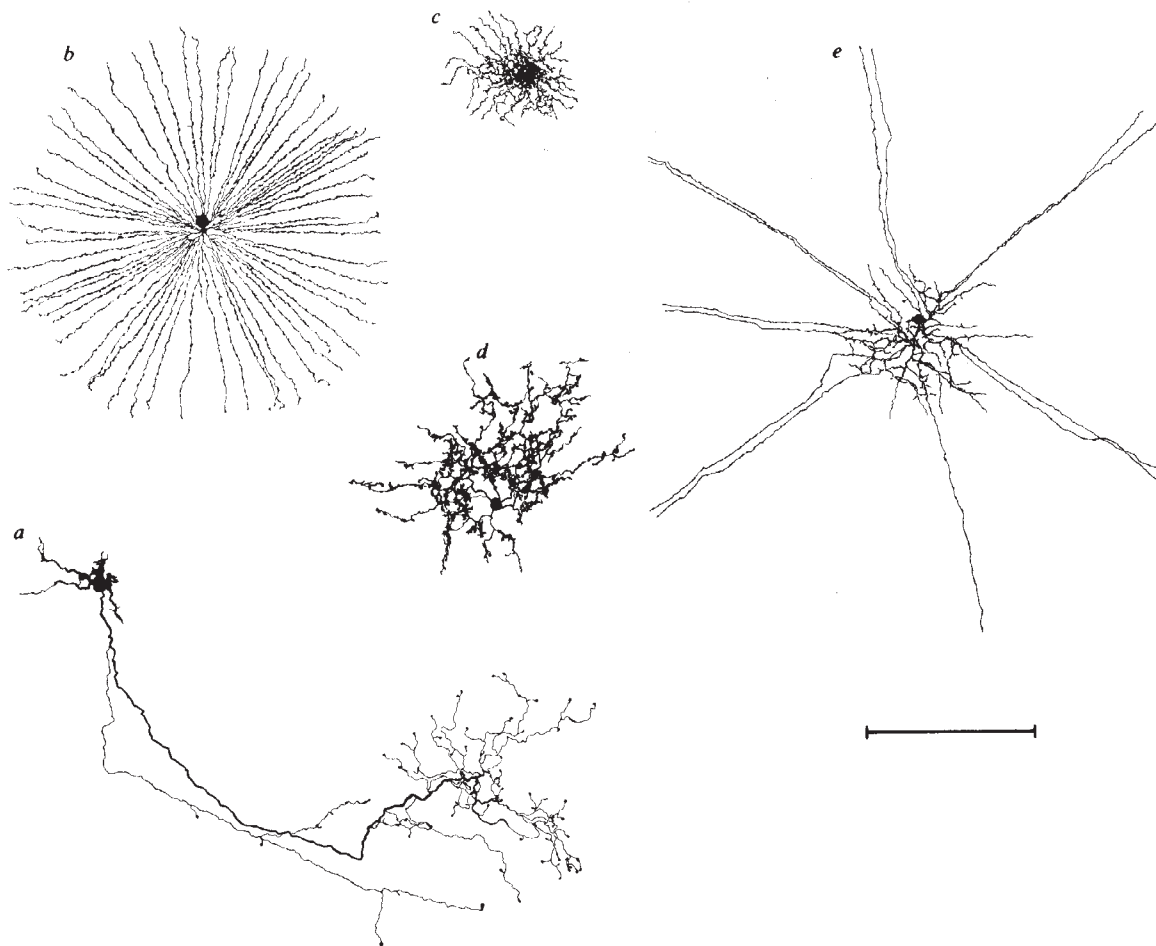


Fig. 1 Camera lucida drawings of Golgi-impregnated amacrine cells in whole, flat-mounted pigeon retina. 'Ordinary' amacrine cells (*b-e*) lack an axon and their dendrites display radial symmetry. 'Association' amacrine cells (*a*) clearly have an intraretinal axon which separates the dendritic end and the axon terminal arborization, and thus are morphologically polarized. This morphological polarization could mediate directional selectivity in pigeon retina. Bar, 100 μ m.

concept of asymmetric lateral inhibition extending in only one direction which determines the null-preferred axis¹¹. Like different directionally selective units, the axons of association amacrine cells have different orientations. The axon terminal arborization could be located over the receptive field centre of the ganglion cell. The soma and dendrites of the cell are then offset from the field centre, and activation of the association amacrine by motion in the null direction as a patch offset from the field centre could inhibit the ganglion cell. Thus motion in the preferred direction could excite the ganglion cell. This model can account for the non-directionality of responses at the receptive field centre and the non-directionality of inhibition which appears to be offset from the receptive field centre and determines the null direction³.

Glycine receptor alteration in the mutant mouse *spastic*

W. F. White* & A. H. Heller†

Department of Neuroscience, Children's Hospital Medical Center and Departments of *Neuropathology and †Neurology, Harvard Medical School, Boston, Massachusetts 02115, USA

The mutant mouse *spastic*, which carries a single-locus, recessive mutation¹ on chromosome 3 (refs 2, 3), is characterized by hyperexcitability, rapid tremor, rigidity and prolonged righting reflexes¹. No anatomical abnormalities have been found in preliminary histological studies of muscle or the central nervous system (CNS)¹. Electromyographic studies in *spastic* mice demonstrate abnormal electrical bursts during activity and abnormal stereotyped reflexes⁴. Drugs which increase γ -aminobutyric acid (GABA)⁵ or enhance GABA synaptic action⁶ reduce *spastic* symptoms, suggesting that they result from an imbalance in excitatory and inhibitory influences in the *spastic* CNS. Strychnine antagonizes the synaptic action of glycine⁷, and binds to a membrane site with the characteristics of the postsynaptic glycine receptor⁸⁻¹¹. The behavioural and electrophysiological abnormalities of *spastic* mice are reproduced in normal mice given subconvulsive doses of strychnine⁴, suggesting that *spastic* symptoms might result from a deficiency in glycine-mediated inhibition⁴. We describe here evidence that supports this hypothesis: a decrease in ³H-strychnine binding in membrane fractions prepared from *spastic* compared with littermate control mice. We also demonstrate an involvement of the benzodiazepine, and possibly the GABA, binding site in the *spastic* phenotype.

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Adult mice of both sexes were produced on site from matings between B6C3-a/a-*spa*/+ hybrids (Jackson Laboratory) and homozygous *spa* mice (*spa*/*spa*) were identified by tremor and difficulty in regaining upright posture when turned on their backs. Unaffected littermates (+/?) of the same sex were used as controls in all studies. *Spastic* mutants are significantly smaller than control animals: a sample of adult *spastic* animals ($n = 7$) weighed $81.7 \pm 4.4\%$ (mean $\pm$ s.e.m.) of their littermate controls and the *spa*/*spa* brain regions (spinal cord, brain stem, midbrain and cortex) weighed an average of $88.4 \pm 8.4\%$ of comparable +/? regions. There were no significant differences among the *spastic* to control weight ratios for the individual regions.

The binding of ^3H -strychnine in *spa*/*spa* and +/? animals is presented in Table 1. In all regions tested, specific ^3H -strychnine binding is reduced in *spa*/*spa* compared with +/?. The low level of binding seen in the *spastic* CNS makes accurate quantification difficult.

Because only a small amount of ^3H -strychnine binding in *spastic* animals is specific, we used several methods for determining nonspecific binding: excess glycine (10 mM) (Table 1), excess strychnine (100 μM), or heat inactivation (80 °C for 30 min) (data not shown). We find that ^3H -strychnine binding is <20% of control in the *spastic* spinal cord, brain stem and midbrain, using any of the measures of nonspecific binding, and <35% of control in cortex using excess glycine to determine nonspecific binding; neither the excess strychnine nor the heat inactivation methods for determining nonspecific binding yielded any specific binding in cortex.

Very different results are obtained in binding studies of the GABA, benzodiazepine and muscarinic acetylcholine receptors (Table 2). Generally, there is more specific ^3H -GABA binding in the spinal cord and brain stem of *spastic* compared with

Table 1 Specific binding of ^3H -strychnine to the CNS of *spastic* and littermate control mice

Region	Genotype	% Specific binding	pmol per mg protein	% Of paired littermate control
Spinal cord	+/?	68	1.26 $\pm$ 0.08	
	<i>spa</i> / <i>spa</i>	29	0.22 $\pm$ 0.06*	16 $\pm$ 4*
Brain stem	+/?	87	1.79 $\pm$ 0.11	
	<i>spa</i> / <i>spa</i>	45	0.34 $\pm$ 0.04*	19 $\pm$ 2*
Midbrain	+/?	60	0.60 $\pm$ 0.04	
	<i>spa</i> / <i>spa</i>	28	0.086 $\pm$ 0.031*	14 $\pm$ 5*
Cortex	+/?	23	0.14 $\pm$ 0.02	
	<i>spa</i> / <i>spa</i>	5	0.047 $\pm$ 0.009*	34 $\pm$ 6*

Mice were decapitated and the brains and spinal cords rapidly dissected, weighed, homogenized in a glass-glass homogenizer in ice-cold 200 mM KCl, 50 mM NaKH₂PO₄, pH 7.1, and centrifuged at 100,000g for 20 min. The pellet was then washed four times by replacing the supernatant with fresh buffer, sonicating to disperse the pellet and recentrifuging. The final pellet was resuspended at a concentration of 5 mg (original wet wt) per ml for use in the binding assays. In ^3H -strychnine binding studies, 500 μl of tissue were added to microcentrifuge tubes (Biorad) containing 500 μl of buffer and 12 nM ^3H -strychnine. Nonspecific binding was determined in parallel incubations containing excess glycine (10 mM). Following a 20 min incubation at 4 °C the samples were centrifuged for 10 min in an Eppendorf microfuge. The supernatant was carefully removed and the pellet washed twice with 1 ml of buffer. The pellet was solubilized overnight in 1 ml 1% SDS, 20 mM EDTA, adjusted to pH 8.5 with NaOH, and counted in a Triton X-100, toluene, Omnifluor liquid scintillation cocktail. Parallel studies of ^3H -strychnine binding using excess strychnine (100 μM) and heat inactivation (80 °C for 30 min) to determine nonspecific binding values were also performed (data not shown). A small amount of ^3H -strychnine was displaced from the assay tube by 100 μM strychnine. Thus, when excess strychnine was used to determine nonspecific binding, nonspecific binding equalled the binding in the presence of excess strychnine plus the binding displaced from the tube. The ^3H -strychnine (19.3 Ci mmol⁻¹) was purchased from Amersham. All experiments on a given sample were performed at least in triplicate and samples were obtained from at least four pairs of animals. Specific binding equals total binding minus nonspecific binding. Per cent specific binding equals specific binding divided by total binding times 100. Proteins were assayed by the method of Bradford²⁵. Values are the mean $\pm$ s.e.m. and all values reported are significantly different from 0. Percentage values are the mean $\pm$ s.e.m. of the percentages derived from comparisons between littermate pairs.

* *Spastic* animals differ from paired littermate control (+/?) at $P \leq 0.05$; Student's *t*-test for paired observations; two-tailed.

Table 2 Specific binding of ^3H -GABA, ^3H -flunitrazepam and ^3H -QNB to the CNS of *spastic* and littermate control mice

Region	Genotype	% Specific binding	pmol per mg protein	% Of paired littermate control
^3H -GABA binding ($n = 6$)				
Spinal cord	+/?	10	0.068 $\pm$ 0.014	
	<i>spa</i> / <i>spa</i>	10	0.089 $\pm$ 0.028	132 $\pm$ 27
Brain stem	+/?	15	0.12 $\pm$ 0.03	
	<i>spa</i> / <i>spa</i>	20	0.16 $\pm$ 0.02	165 $\pm$ 34
Midbrain	+/?	39	0.37 $\pm$ 0.03	
	<i>spa</i> / <i>spa</i>	43	0.35 $\pm$ 0.04	96 $\pm$ 10
Cortex	+/?	61	0.92 $\pm$ 0.04	
	<i>spa</i> / <i>spa</i>	61	0.93 $\pm$ 0.08	101 $\pm$ 7
^3H -flunitrazepam binding ($n = 4$)				
Spinal cord	+/?	74	0.45 $\pm$ 0.03	
	<i>spa</i> / <i>spa</i>	84	0.62 $\pm$ 0.03*	140 $\pm$ 8*
Brain stem	+/?	87	0.85 $\pm$ 0.02	
	<i>spa</i> / <i>spa</i>	90	0.99 $\pm$ 0.02*	117 $\pm$ 5*
Midbrain	+/?	94	2.17 $\pm$ 0.06	
	<i>spa</i> / <i>spa</i>	95	2.20 $\pm$ 0.01	102 $\pm$ 3
Cortex	+/?	95	2.87 $\pm$ 0.04	
	<i>spa</i> / <i>spa</i>	94	2.77 $\pm$ 0.12	96 $\pm$ 4
^3H -QNB binding ($n = 4$)				
Spinal cord	+/?	71	0.92 $\pm$ 0.06	
	<i>spa</i> / <i>spa</i>	72	0.93 $\pm$ 0.05	102 $\pm$ 8
Cortex	+/?	91	5.14 $\pm$ 0.28	
	<i>spa</i> / <i>spa</i>	90	5.22 $\pm$ 0.33	102 $\pm$ 4

A centrifugation assay similar to that used for the strychnine binding assays was used for the GABA binding studies. The tissues used in the GABA binding assay were prepared in Tris-citrate buffer (4 °C, pH 7.1) by washing four times followed by three freeze-thaw-wash cycles. The equivalent of 10 mg of tissue (original wet weight) in 1 ml was used in each tube. The ^3H -GABA concentration was 30 nM, and 1 mM GABA was used to determine nonspecific binding. A 10 min centrifugation followed the 10 min incubation. Pellets were washed twice with 1 ml ice-cold Tris-citrate (pH 7.1), solubilized and counted. The binding of both ^3H -flunitrazepam and ^3H -QNB was investigated using a filtration assay. Briefly, 500 μl of the same tissue suspension used for the strychnine binding studies were added to 500 μl of buffer containing either ^3H -flunitrazepam (1 nM) or ^3H -QNB (1 nM). Clonazepam (1 μM) or atropine (1 μM) were added to parallel incubations to determine nonspecific binding. Following a 1 h incubation (4 °C flunitrazepam, 24 °C QNB) the bound radioactivity was collected on a 24 mm glass fibre filter (Whatman GF-B) and washed three times with 5 ml of ice-cold 200 mM KCl buffered to pH 7.1 with 50 mM NaKH₂PO₄. The filters were air-dried and counted in liquid scintillation cocktail. The ^3H -GABA (34.3 Ci mmol⁻¹), ^3H -flunitrazepam (86.4 Ci mmol⁻¹) and ^3H -QNB (29.1 Ci mmol⁻¹) were from NEN. See Table 1 legend for further details.

control animals. However, because of the low level of specific ^3H -GABA binding (10–20%) in these two brain regions, the results were relatively more variable, and the observed difference was not statistically significant ($P > 0.05$). Specific binding of ^3H -flunitrazepam is 40% greater in the spinal cord, and 17% greater in the brain stem of *spastic* compared with control animals ($P < 0.05$, both regions); again there are no differences in the midbrain and cortex. These increases in binding to the benzodiazepine binding site and potentially to the GABA binding site are consistent with preliminary studies using ^3H -flunitrazepam and ^3H -muscimol as ligands for the benzodiazepine and GABA binding sites¹². In contrast, there are no significant differences in the binding of ^3H -QNB (quinuclidinyl benzilate) in any of the regions investigated. The magnitude and regional distribution of the binding of all four ligands in control animals are similar to published values for rodent brain^{8,13–15}.

The alterations in the binding of ligands for several neurotransmitters in *spa*/*spa* mice is very provocative. The very low binding of ^3H -strychnine in the *spa*/*spa* CNS demonstrates an alteration in the receptor for glycine, the major inhibitory neurotransmitter in spinal cord and brain stem, and a significant inhibitory neurotransmitter in midbrain⁷. This alteration might be expected to decrease the efficacy of glycine-mediated inhibition in these brain areas. On the other hand, the trend towards

greater binding of ^3H -GABA to the GABA receptor and the greater binding of ^3H -flunitrazepam to the benzodiazepine binding site (which is believed to modulate the GABA receptor¹⁶) in the spinal cord and brain stem would potentially increase the efficacy of inhibition mediated by GABA, the other major inhibitory neurotransmitter in these brain areas⁷. It is tempting to speculate that this alteration in the GABA-benzodiazepine system represents a compensatory response to the decrease in glycine-mediated inhibition. The ability of drugs that enhance GABA-mediated inhibition to reduce the severity of the behavioural⁵ and electromyographic⁶ symptoms of the *spastic* mutant mouse is consistent with this hypothesis. The localization of the increase in GABA and benzodiazepine binding to the spinal cord and brain stem may result from an inability of the normal GABAergic tone to regulate neuronal excitability in these regions when their normal preponderance of glycine-mediated inhibition is reduced. The absence of this effect in the midbrain and cortex may result from the secondary role of glycine-mediated inhibition in these brain areas and thus the availability of sufficient GABAergic tone to control neuronal excitability. The lack of any difference in the binding of ^3H -QNB to the muscarinic acetylcholine receptor in any of the brain regions tested indicates specificity in the alterations produced by the *spastic* mutation.

Alterations in neurotransmitter receptors have been reported for other murine mutants, and have been attributed to morphological changes in the mutant animal. Both *nervous* and *staggerer* mice, mutants which have a reduced number of Purkinje cells and lack synaptic spines on Purkinje cells respectively, have fewer benzodiazepine binding sites in cerebellum than controls¹⁷⁻²⁰. The opposite situation—a greater number of cerebellar benzodiazepine binding sites—has been reported for *weaver* and *reeler* mice, mutants having a reduced number of cerebellar granule cells^{17,20}. Fewer GABA receptors have also been reported for all these cerebellar mutants^{20,21}.

Unlike the cerebellar mutants, the *spastic* mutant shows no obvious anatomical abnormalities in either nervous system or musculature in preliminary histological studies¹. In addition, the widespread distribution of binding sites for GABA, benzodiazepines and strychnine in the spinal cord, brain stem and midbrain²²⁻²⁴ makes it seem improbable that the alterations could result from an anatomical abnormality. These preliminary anatomical studies must, however, be confirmed by further, more detailed investigations on the morphological organization of the *spastic* CNS, and in particular of proposed glycinergic and GABAergic inhibitory systems in the affected areas.

These results, together with those from electromyographic studies⁴, indicate an alteration in the glycine system in the *spa/spa* mouse which is the probable explanation of the behavioural and electromyographic abnormalities in these mice. They also demonstrate an involvement of the benzodiazepine binding site, and possibly the GABA binding site, in the expression of the *spastic* mutation. The molecular basis of the mutation in the *spastic* mouse which produces these observed alterations in the receptors for neurotransmitters mediating CNS inhibition is unknown. The mutation may result in a direct alteration in one of the receptors, in a component directly associated with one of the receptors, or in some other aspect of one of the affected neurotransmitter systems. On the other hand, the observed effects may be only a few of many widespread secondary effects produced by the mutation. A more complete characterization of both the glycine and GABA-benzodiazepine inhibitory systems in *spa/spa*, proven heterozygous and +/- animals will further clarify the relationship between these systems and the mutation, and may elucidate the nature of the mutation. The *spastic* mutant mouse should be a valuable system for future investigations on the characteristics of CNS inhibition.

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Aspartate aminotransferase-like immunoreactivity as a marker for aspartate/glutamate in guinea pig photoreceptors

Richard A. Altschuler*, Judith L. Mosinger†*, George G. Harmison*, Marianne H. Parakkal* & Robert J. Wenthold*

* Laboratory of Neuro-otology, National Institute of Neurological and Communicative Disorders and Stroke, National Institutes of Health, Bethesda, Maryland 20205, USA

† Department of Cellular and Developmental Biology, Harvard University, Cambridge, Massachusetts 02138, USA

Immunocytochemical techniques have increased our ability to identify neurones containing a specific neurotransmitter. These techniques could not, however, be applied to two major neurotransmitter candidates, aspartate and glutamate, until our recent study in which aspartate aminotransferase (L-aspartate:2-oxoglutarate aminotransferase, EC 2.6.1.1, AATase) was proposed as a marker¹. This enzyme catalyses the reversible reactions of aspartate to oxaloacetate and α -ketoglutarate to glutamate. In the auditory nerve, which is considered to be aspartergic/glutamergic²⁻⁴, AATase was immunocytochemically localized in primary afferent terminals in the cochlear nucleus¹. These results suggest a presynaptic enrichment of AATase is associated with aspartergic/glutamergic neurotransmitter function. We have now investigated the distribution of AATase-like immunoreactivity in the retina where previous work suggests that glutamate or aspartate is the neurotransmitter of photoreceptors⁵⁻¹⁴. Our results, reported here, show AATase-like immunoreactivity localized in cones and their terminals in the guinea pig retina.

We used 12 female NIH strain guinea pigs, 150-250 g, in this study. Six guinea pigs were pretreated with colchicine: 10 μg in 5 μl were injected into the vitreous of the left eye 24-48 h before perfusion in guinea pigs under ether anaesthesia. Guinea pigs were anaesthetized with chloral hydrate (0.3 g per kg body weight) and perfused through the heart with 25 ml 0.1 M sodium cacodylate buffer followed by 500 ml of fixative containing 4% paraformaldehyde (w/v) in 0.1 M sodium cacodylate buffer, pH 7.2 at 4°C. In some animals, 50 μl of the fixative were injected intravitreally immediately before vascular perfusion. Retinae from both eyes were placed in the same fixative for 1 h on ice and then rinsed in phosphate-buffered saline (PBS) pH 7.2, with 6% (w/v) sucrose at 4°C for 16-48 h. Ten- μm cryostat sections were cut through the central and peripheral retina and the indirect

immunofluorescence technique of Coons¹⁵, modified as described previously¹, was applied using antiserum, raised in rabbits, to cytoplasmic AATase¹. Sections were incubated for 18 h at 4 °C with antiserum diluted 1:400 in PBS with 0.3% Triton X-100. Absorption controls were prepared by adding 20 µg of AATase (Boehringer Mannheim) to 10 µl of antiserum and were used at a 1:400 dilution on adjacent sections as a control for specificity of staining. The second incubation was carried out with fluorescein-labelled swine antiserum to rabbit immunoglobulin (Bio-Rad) diluted 1:10, for 30 min at 37 °C. Sections were viewed with a Zeiss photomicroscope under epifluorescent illumination.

In the retinae of animals without colchicine pretreatment, a band of AATase-like immunofluorescence was seen in the outer plexiform layer (OPL; Fig. 1a). In absorption controls, as in the AATase-immunostained preparations, the inner segments of photoreceptors fluoresced non specifically. This was the only fluorescence seen in the retina on absorption control sections (Fig. 1b). Colchicine has often been used to concentrate particular antigens in cell bodies¹⁶ and we therefore used colchicine to determine the source of the band of immunofluorescence in the OPL. In retinae pretreated with colchicine, this band could more easily be resolved into individual swellings (Fig. 2c). AATase-like immunofluorescence also was seen in cell bodies, in the outer portion of the outer nuclear layer (ONL), giving rise to thin processes ending in large swellings in the OPL (Fig. 2c,d). This is consistent with the morphological criteria of cones and cone pedicles¹⁷.

In retinae with and without colchicine pretreatment, AATase-like immunoreactivity was often seen in a subpopulation of cells along the innermost margin of the inner nuclear layer (INL; Fig. 2a-c) and in a laminar pattern in the inner plexiform layer (IPL; Figs 1a, 2a-d). These are likely to be a subpopulation of amacrine cells and their fibres.

Immunoreactive cells were also seen in the ganglion cell layer (GCL; Fig. 2a), though much less frequently than cells in the IPL. These were easiest to identify in retina prefixed intravitreally due to the better preservation of the ganglion layers. The immunoreactive cells in the GCL might be displaced amacrine cells or may correspond to the class of ganglion cells in which selective uptake of tritiated D-aspartate has been described^{11,18}.

Several lines of evidence have suggested that aspartate and/or glutamate is a vertebrate photoreceptor transmitter. Physiological studies have shown that glutamate and aspartate mimic the effects of the natural photoreceptor on horizontal and bipolar cells⁵⁻⁹ and these effects can be eliminated by excitatory amino acid inhibitors^{9,10}. Recently Ehinger¹¹ has demonstrated uptake of D-aspartate into photoreceptors, probably cones, in guinea pig, rabbit and pigeon. Uptake of aspartate and glutamate has also been shown in goldfish photoreceptors¹²

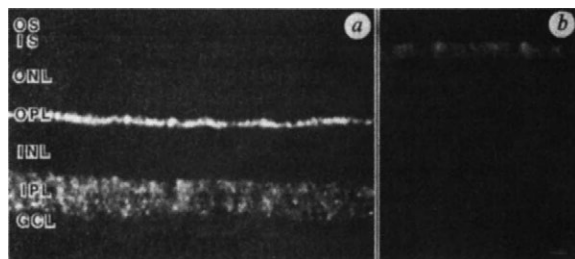


Fig. 1 Fluorescence micrographs of 10 µm cryostat sections of guinea pig retina incubated with antiserum to AATase (a) or to AATase preabsorbed with AATase (b). a, Section showing a band of AATase-like immunofluorescence in the outer plexiform layer and immunofluorescent fibres in the inner plexiform layer. b, Absorption control section showing nonspecific fluorescence in inner segments of photoreceptors. OS, outer segments; IS, inner segments; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Scale bar, 20 µm.

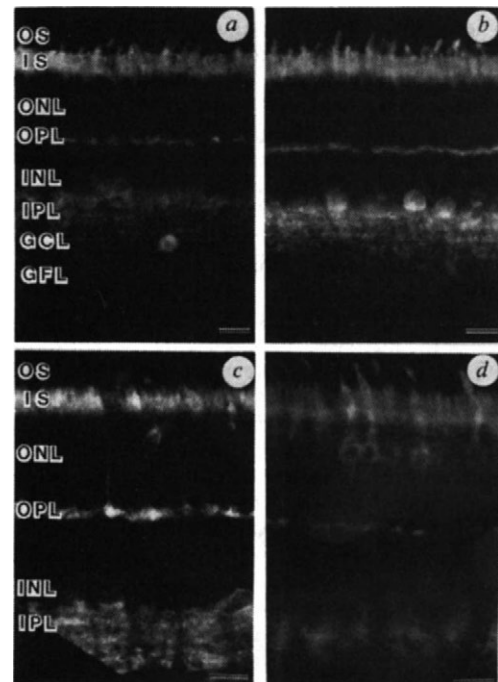


Fig. 2 Fluorescence micrographs of 10 µm cryostat sections of guinea pig retina incubated with antiserum to AATase. a, Section through central retina showing AATase-like immunoreactivity in the outer plexiform layer and immunofluorescent cells in the inner nuclear layer and ganglion cell layer. b, Section through central retina showing a band of AATase-like immunoreactivity in the outer plexiform layer, immunofluorescent cells in the inner nuclear layer and immunofluorescent fibres in the inner plexiform layer. c, Section through retina pretreated with colchicine as described in the text. AATase-like immunoreactivity can be seen in a cone cell body and a cone process and terminal, and immunofluorescent cell bodies and fibres in the inner nuclear and inner plexiform layers. d, Section through non-colchicine pretreated retina showing immunofluorescent cones and cone processes. OS, outer segments; IS, inner segments; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer; GFL, ganglion fibre layer. Scale bars, 20 µm.

where both amino acids were found to be concentrated in red and green cones while only glutamate was concentrated in rods. In rabbit retina, aspartate release has been found to decrease in response to light as would be expected of the natural photoreceptor¹³. Our findings of AATase-like immunoreactivity in cones and cone pedicles provide further evidence that aspartate or glutamate is the neurotransmitter of cones. A recent study showing L-aspartate enhanced excitation of sustained ganglion cells in the cat^{19,20} is consistent with our finding of AATase-like immunoreactivity in amacrine cells and suggests aspartate or glutamate as a transmitter for a class of amacrine cells. Ultrastructural immunocytochemical studies are in progress to determine whether AATase is enriched in rod terminals as well as cones and to characterize the immunoreactive cells and fibres in the INL and IPL.

The present results support our hypotheses that aspartate aminotransferase can serve as a marker for synapses using aspartate or glutamate as a neurotransmitter. While it is a ubiquitous enzyme, aspartate aminotransferase is enriched in terminals of cones where it may be involved in the specialized role of neurotransmitter synthesis.

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Origin of lignans in mammals and identification of a precursor from plants

M. Axelson*, J. Sjövall*, B. E. Gustafsson† & K. D. R. Setchell‡

* Department of Physiological Chemistry and † Department of Germfree Research, Karolinska Institutet, S-104 01 Stockholm, Sweden

‡ Division of Clinical Chemistry, Clinical Research Centre, Harrow, Middlesex HA1 3UJ, UK

Lignans are a class of compounds with a dibenzylbutane skeleton¹ which until recently had only been found in higher plants^{2,3}. The first lignans to be identified in humans and animals were *trans*-2,3-bis(3-hydroxybenzyl)- γ -butyrolactone (enterolactone)⁴⁻⁶ and 2,3-bis(3-hydroxybenzyl)butane-1,4-diol (enterodiol)^{4,5}. These structures differ from those of plant lignans by having aromatic rings substituted only in the *meta* position. Enterolactone and enterodiol are excreted in the urine and bile, predominantly as glucuronide conjugates^{7,8}, and intestinal microflora is required for their formation^{8,9}. It is not known, however, whether they are synthesized *de novo* by microorganisms or are formed from dietary precursors. We report here evidence that these mammalian lignans are formed by microbial action on precursor lignans which are present as dietary constituents of plant origin. Precursors are found in seeds of different species, being particularly abundant in linseed. From this source, 2,3-bis(3-methoxy-4-hydroxybenzyl)butane-1,4-diol (secoisolariciresinol) has been identified as a glycoside.

Evidence for dietary precursor(s) was first obtained by changing the diet given to adult rats from commercial pellets to a semisynthetic diet, D7 (ref. 10), which resulted in a marked and rapid decrease in the excretion of mammalian lignans in urine (Fig. 1). When the diet was reverted to commercial pellets, lignans reappeared in the urine.

The minimum concentration of lignan precursors in different food constituents was determined by adding the latter to the semisynthetic diet and measuring the total excretion of lignans in urine. In decreasing order, rye, buckwheat, millet, soya, oat and barley gave from 6 to 2 μ g of lignans in urine per g of meal added. Wheat bran gave about 8 μ g per g, wheat and corn meal gave less than 1 μ g per g, while linseed was by far the richest source of precursor(s), giving about 800 μ g per g of meal. The corresponding value for linseed oil was only 17 μ g per g.

Preliminary experiments to isolate the precursor(s) from barley, rye, wheat bran and linseed revealed that the compound(s) were polar and could be extracted with 80% aqueous methanol and separated from fibre. After removal of salts and lipids and fractionation on a column of TEAP-LH-20 [OH]⁻, about 70% of the precursor(s) in linseed was found in the phenolic fraction. Further separation on DEAE-Sephadex¹¹ yielded more than 80% of the precursor(s) in the diphenolic fraction. Analytical TLC showed a major highly polar component. Mass spectra of

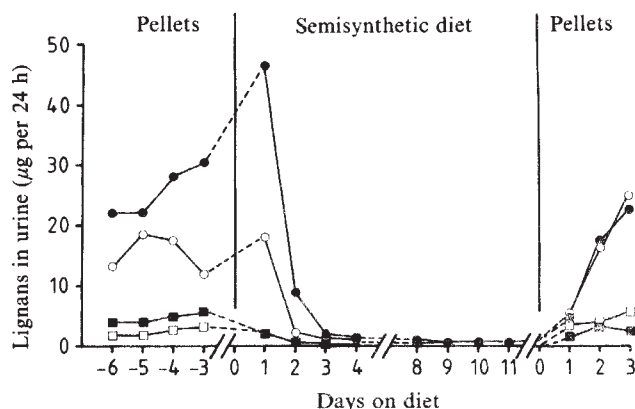


Fig. 1 Daily urinary excretion of enterolactone (circles) and enterodiol (squares) by two adult Long Evans rats (female, body weight 200 g) fed commercial pellets (Astra Ewos, Sweden) or a semisynthetic diet, D7 (ref. 10), composed of wheat starch, casein, arachis oil, salts and vitamins. Lignans were purified and measured by gas chromatography^{7,8,11}.

the trimethylsilyl (TMS) ether derivative were recorded using a direct probe. Peaks at *m/z* 451, 361 (base peak), 319, 271, 243, 217 and 204 were indicative of a TMS ether of a hexoside¹². An intense peak at *m/z* 209 suggested a benzyl group substituted with a methoxy and a trimethylsiloxy group¹³. This interpretation was supported by the mass spectrum of the perdeuterated TMS ether derivative. Hydrolysis with β -glucosidase (Emulsin, Sigma) yielded a compound the TMS ether of which had the same retention time on SE-30 (1.54 relative to 5 α -cholestane) and OV-17 and the same mass spectrum as the TMS ether of a reference sample of secoisolariciresinol. The fragmentation of the latter compound has been discussed previously¹³. The TLC mobilities of the non-derivatized compounds were also identical. When the reference compound (300 μ g) was added to the semisynthetic diet for 1 day, approximately 85 μ g of lignans of the mammalian type, predominantly enterolactone, were excreted in the urine, confirming that secoisolariciresinol is the major precursor of enterolactone in rats given linseeds.

Extending these studies to man, the 'normal' diet of adults was supplemented with linseeds and the urinary excretion of enterolactone and enterodiol measured. The response to this diet was analogous to that observed in rats, indicating that the precursor-product relationships are similar in man and rats (Fig. 2).

Our results show that the precursor present in linseeds is a hexoside of 2,3-bis(2-methoxy-4-hydroxybenzyl)butane-1,4-diol. The tentative structure proposed in Fig. 3 is based on the previous finding of a diglucoside of a lignan in linseeds¹⁴.

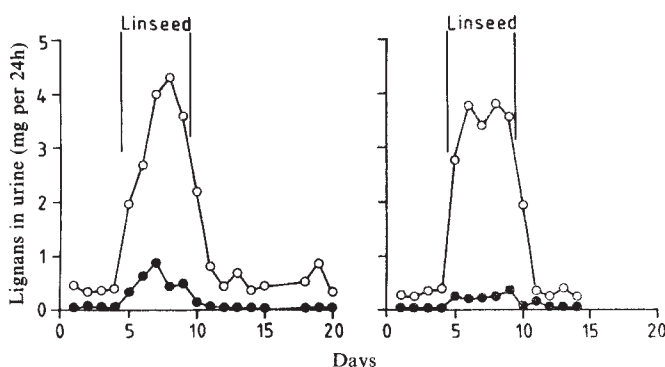


Fig. 2 Daily urinary excretion of enterolactone (○) and enterodiol (●) by two healthy adults before and after the addition of linseeds (10 g per day) to the normal diet over a period of 5 days.

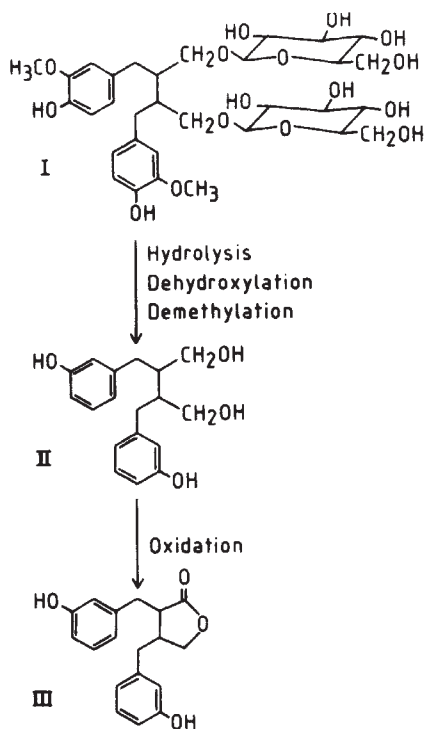


Fig. 3 A simplified scheme for the formation of enterodiol (II) and enterolactone (III) from the dietary precursor secoisolariciresinol glycoside (I). The structure of the sugar moiety is tentative and the exact sequence of reactions is not fully understood.

However, further studies are required to determine the number and position of hexose residues and the stereochemistry of the compound. Other precursors may also exist in plants. Thus, when rats were fed the more abundant plant lignan, matairesinol, enterolactone was excreted in the urine.

Conversion of secoisolariciresinol glycoside to enterodiol requires reactions depicted in Fig. 3. This type of reaction can be carried out by microbial enzymes^{15,16}. Extensive formation of enterolactone from enterodiol was demonstrated by feeding 50 µg of the latter¹⁷ to rats in a semisynthetic diet. Enterolactone was not detected when enterodiol was administered orally to germ-free rats, or injected intraperitoneally to bile fistula rats. This indicates that enterolactone can be formed from enterodiol by microbial action.

Lignans have been extensively investigated for their potential anticancer activity, and several have been used as chemotherapeutic agents^{3,18}. It is therefore of interest that several common foodstuffs contain lignans. The compounds usually exist in optically pure form; however, enterolactone in human urine is racemic⁴⁻⁶. Whether this is due to its formation from precursors of different origin or to isomerization by exchange of a hydrogen vicinal to the carbonyl group of the lactone is unknown. The rate of formation of enterolactone and enterodiol is conceivably influenced by the composition of the microflora, the intestinal transit time and the redox level in the large intestine. Progesterone decreases intestinal motility¹⁹, and this might explain the finding that the excretion of lignans in urine varies during the menstrual cycle^{4,6,20,21}.

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Fibronectin in adhesion, spreading and cytoskeletal organization of cultured fibroblasts

I. Virtanen*, T. Vartio*†, R. A. Badley‡ & V.-P. Lehto*

Departments of Pathology* and Virology†, University of Helsinki, Haartmaninkatu 3, SF-00290 Helsinki 29, Finland

‡ Unilever Research, Colworth Laboratory, Sharnbrook, Bedford MK44 1LQ, UK

The pericellular glycoprotein fibronectin, which has been localized in the pericellular matrix and at the adhesion sites of cells¹⁻⁵, has been considered to be important in cell attachment to other cells and to surfaces¹⁻⁸. Using the ionophore monensin, which blocks the secretion of endogenous fibronectin^{9,10}, we have now shown that human fibroblasts do not require endogenous or exogenous fibronectin for adhesion and spreading, although its absence does prevent formation of the microfilament-vinculin system and the focal adhesion sites.

The indirect immunofluorescence (IIF) technique was used to stain for the presence of fibronectin. Figure 1 shows that trypsinized human embryonal fibroblasts, which adhere to and spread on plastic Petri dishes after plating, develop a typically fuzzy fibronectin-containing plaque after 5 h (Fig. 1a, b) and a fibrillar extracellular network of fibronectin after 24 h in culture (Fig. 1c, d). No cell-surface-associated fibronectin could be detected on monensin-treated cells (Fig. 1e, g) which did, however, adhere and spread in the same way as control cells (Fig. 1f, h). Monensin-treated cells do stain for fibronectin in their cytoplasm (Fig. 1j), and treatment with the protein synthesis inhibitor puromycin, which causes the cytoplasmic staining of control cells to disappear (Fig. 2e), results in an accumulation of fibronectin in the Golgi apparatus (Fig. 2f).

The blockage of fibronectin secretion by monensin is further illustrated in Fig. 3, which shows the lack of a polypeptide of molecular weight (M_r) 220,000 (220 K) which corresponds to fibronectin¹¹, and is the major product in ³⁵S-methionine-labelled control cells, in monensin-treated cells. As shown in Fig. 3b, surface labelling of control cells, using the galactose oxidase/sodium borohydride technique¹², also shows up a 220 K polypeptide as a major glycoprotein, lacking in monensin-exposed cells. After 24 h in normal medium, monensin-treated cells recovered the characteristics of normal cells. The surface labelling results also draw attention to the role of cell membrane proteins in cell adhesion and spreading. Interestingly, recent studies have suggested distinct cell-surface glycoproteins¹³⁻¹⁶ to be important in cell adhesion. A cell-surface glycoprotein of M_r 140 K could also be shown in this study to be a major cell-surface glycoprotein in cells plated and allowed to spread in the presence of monensin (Fig. 3b, asterisk). We have previously shown¹⁴ that the 140 K glycoprotein is the

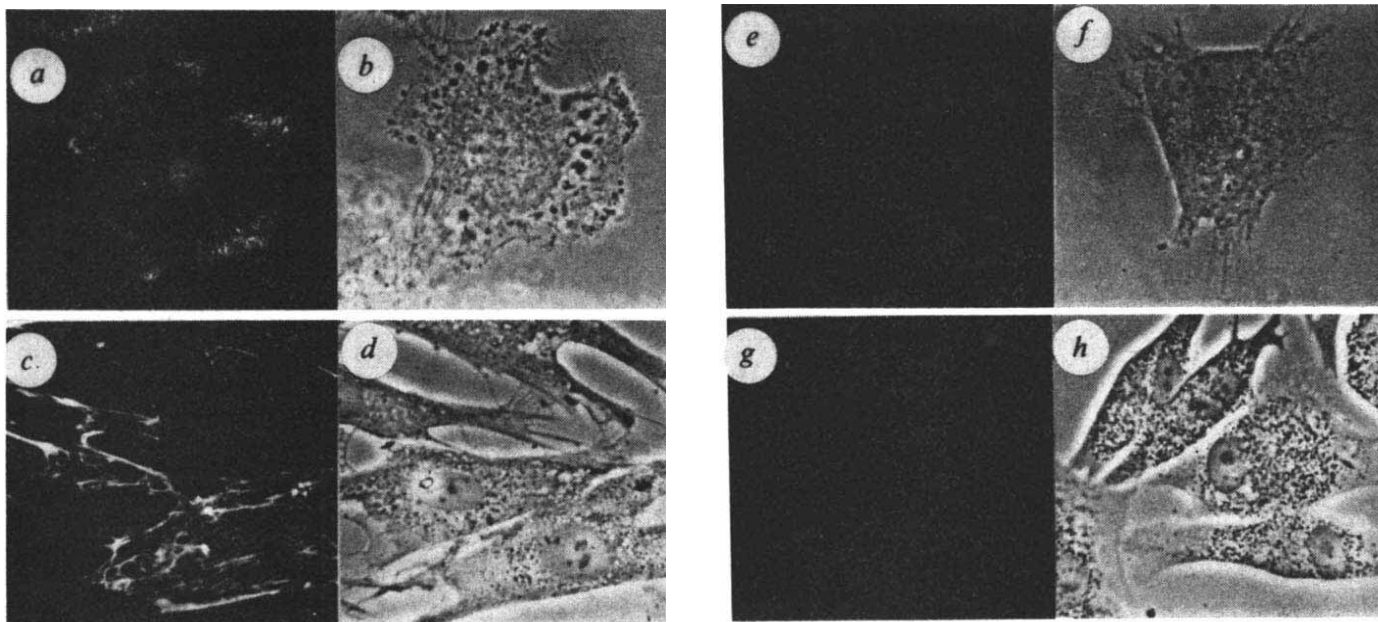


Fig. 1 Cultured human fibroblasts were plated after trypsinization in RPMI 1640 medium supplemented with 10% fetal calf serum and antibiotics. Five hours after plating a fuzzy fibrillar fibronectin-specific staining is seen by the indirect immunofluorescence (IIF) technique on the ventral surface of the cells when focused to the growth substratum level (*a, b*). After 24 h in culture, a typical fibrillar matrix is seen in the cultures with anti-fibronectin antibodies (*c, d*). In spread cells, fibronectin-specific staining is mainly confined to the upper surface of the cells. Similar staining patterns were obtained when the cells were plated in the absence of serum. When plated in the presence of monensin (Lilly; $1 \mu\text{M}$) the cells adhered and spread as well as control cells (compare with *f, h*). No fibronectin-specific fluorescence was seen in these cells in surface staining either after the first 5 h on plating (*e*) or in fully spread cells (*g*). In adhering cells treated with 0.5% Triton X-100¹⁴ at 6 h of spreading, a typical fuzzy fibrillar fibronectin-specific fluorescence is seen beneath the control cells, with some diffuse staining in the cytoplasmic domain (*i*) whereas the cells allowed to spread in the presence of monensin only show the remnant cytoplasmic staining following Triton treatment (*j*). In *j* the cell margins are indicated by arrowheads. For surface staining, unfixed cells were exposed to the first antibody washed in phosphate-buffered saline, fixed in 3.5% paraformaldehyde and then exposed to the second antibody, fluorescein isothiocyanate (FITC)-coupled goat anti-rabbit IgG (Cappel). Rabbit antifibronectin antibodies were obtained from Cappel. *a, b, e, f, i, j*: $\times 800$; *c, d, g, h*: $\times 400$.

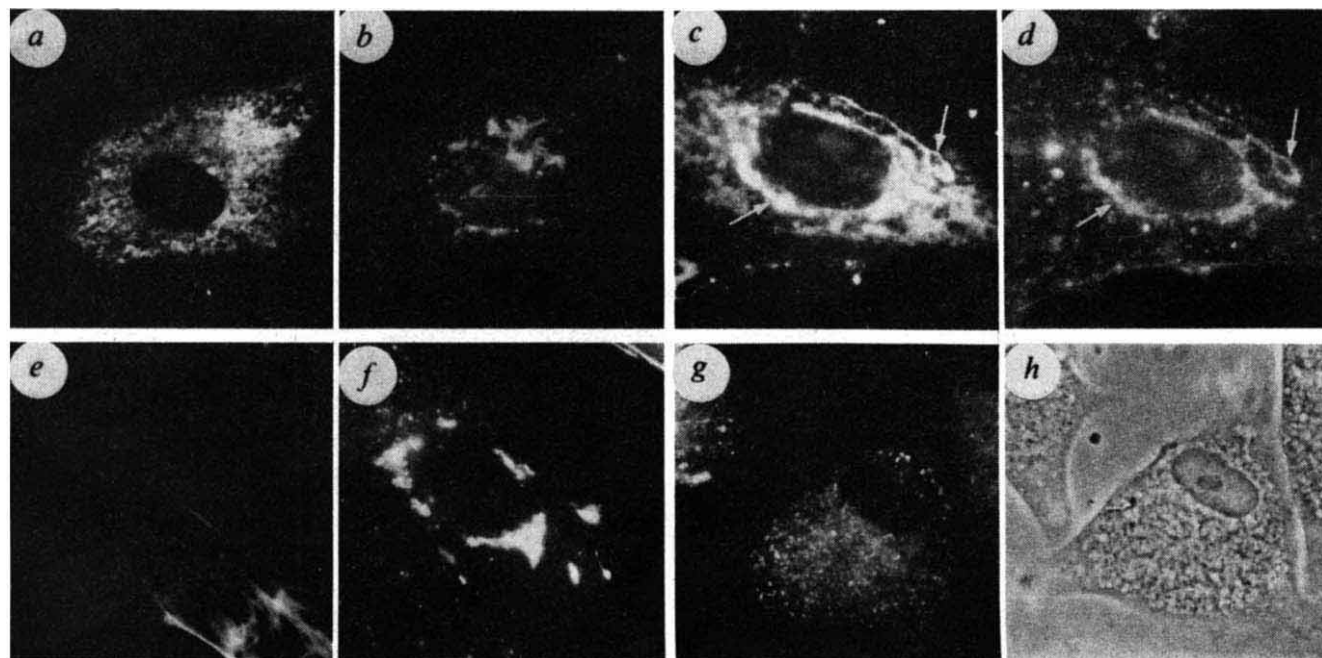


Fig. 2 In control cells fixed in 3.5% paraformaldehyde and permeabilized with 0.1% NP40, a cytoplasmic granular perinuclear staining is seen (*a*). In double-staining with TRITC-wheat germ agglutinin (WGA, Vector Laboratories), used here as a marker for the Golgi apparatus⁴⁰, a distinctly different reticular juxtanuclear staining was seen (*b*). Note that no apparent labelling of the Golgi region is seen in *a*. When the cells were exposed to monensin ($1 \mu\text{M}$) for 1 h, a diffuse cytoplasmic but also a pronounced reticular juxtanuclear fibronectin-specific staining was seen (*c*). The reticular staining (arrows in *c, d*) coincided with that obtained with TRITC-WGA (*d*), marking the Golgi apparatus. After exposure to puromycin ($2 \mu\text{g ml}^{-1}$) for 2 h, no cytoplasmic fluorescence could be detected in control cells (*e*) whereas cells first exposed to monensin ($1 \mu\text{M}$, 1 h) still showed a bright juxtanuclear fibronectin-specific fluorescence after puromycin treatment (*f*). Cells cultured in the presence of monensin for 24 h, on the other hand, showed a granular cytoplasmic accumulation of fibronectin in IIF but no fibrillar matrix-type fibronectin (*g, h*, cells plated in the presence of monensin). $\times 800$.

only surface membrane glycoprotein present in detergent-resistant cytoskeletons of human fibroblasts still anchored to the culture substratum.

With the IIF technique using anti-actin (Fig. 4a) and anti-vinculin (Fig. 4b) antibodies control cells showed the typical organization of stress fibres and vinculin plaques. Interference reflection microscopy revealed 'black' arrays of focal adhesions in control cells (Fig. 5a). In contrast to control cells, IIF of cells plated in the presence of monensin showed only a diffuse actin- and vinculin-specific staining (Fig. 4c, d). They displayed, however, typical fibrillar arrays of both vimentin-type intermediate filaments and microtubules (Fig. 4e, f). Monensin-exposed cells did not exhibit focal adhesion sites in interference reflection microscopy but instead showed large grey-coloured areas representing sites of close adhesion (Fig. 5d). However, cells first spread in normal medium and then exposed to monensin showed normal arrays of both microfilament bundles (Fig. 4g, h) and vinculin plaques. A mouse fibroblastoid cell line (3T3), which is known to require exogenous fibronectin for both adhesion and spreading¹, spread on exposure to monensin only if serum was present, but did not develop stress fibres or vinculin plaques (data not shown).

Focal adhesions are specialized cell-surface areas of cell-to-substratum adhesion of cultured cells¹⁷⁻²⁰ and emerge concomitantly with the formation of bundles of microfilaments^{18,19} during cell spreading. Both the vinculin plaques^{21,22} and the focal contacts^{17,22,23} have been reported to be in close apposition to pericellular fibronectin, and isolated focal adhesions have been reported to contain both actin and fibronectin¹⁷. During cell spreading, fibronectin seeks to co-align closely with microfilaments²³⁻²⁵. Transformed cells, lacking cell-surface-associated fibronectin^{4,5}, also lack bundles of microfilaments^{26,27}, but normal arrays of microfilaments are restored on addition of cell-derived fibronectin²⁸. However, recent results²⁹⁻³¹ have indicated that fibronectin might be absent or even actively removed from focal adhesions. Based on such an

observation, Chen and Singer²⁹ recently proposed two different molecular mechanisms for microfilament-cell membrane interaction in cultured cells, involving fibronectin differently in cytoskeletal organization and adhesive functions of cultured cells.

Our results with monensin-exposed cells suggest that fibronectin is not needed for fibroblast adhesion to and spreading on culture substrata. On the other hand, endogenous fibronectin seems to be required for the development of focal

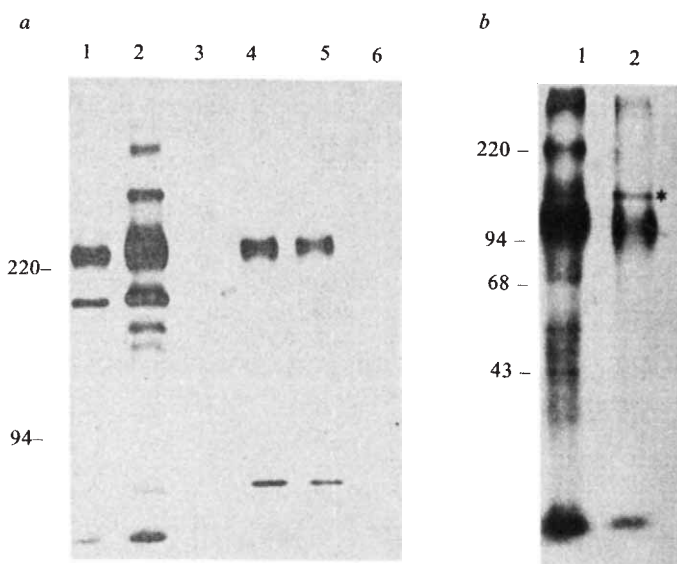


Fig. 3 a, Electrophoretic analysis of secreted polypeptides in the culture medium of control cells labelled with L-³⁵S-methionine for 24 h (1,000 Ci mmol⁻¹; 10 μ Ci ml⁻¹; Radiochemical Centre) showed several polypeptides both after 5 h and 24 h of plating (lanes 1, 2) whereas cultures plated in the presence of monensin and cultured for 24 h did not contain any secreted proteins (lane 3). A prominent 220 K gelatin-binding polypeptide, corresponding to fibronectin¹¹, was seen in the medium of both spreading (5 h, lane 4) and wholly spread control cultures (24 h, lane 5), but not in cultures plated in the presence of monensin (24 h, lane 6). b, Surface labelling experiments using the neuraminidase/galactose oxidase/sodium borohydride method¹² showed a distinct polypeptide with *M_r* 220 K and several polypeptides of lower molecular weight in control cultures (lane 1). Instead, cells exposed to monensin during plating lacked the 220 K glycoprotein but showed a prominent 140 K glycoprotein (asterisk in lane 2) together with some other polypeptides (lane 2).

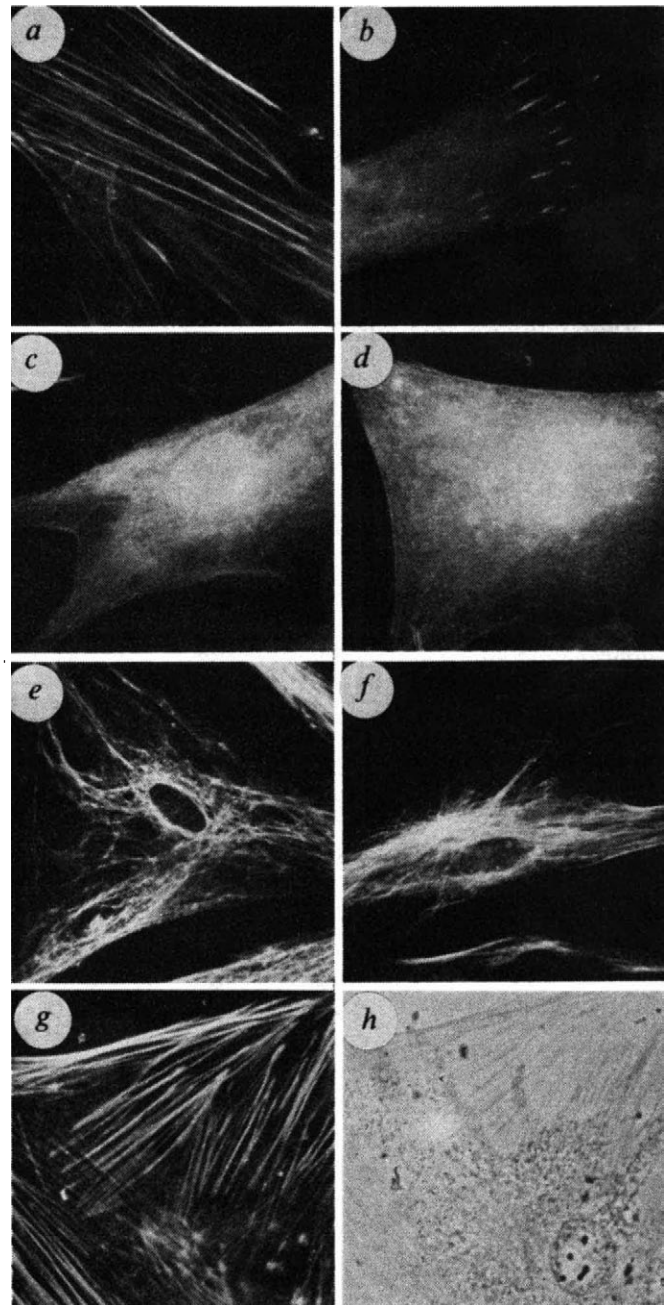


Fig. 4 Control human embryonal fibroblasts showed a stress fibre-type of actin organization in IIF with anti-actin antibodies¹⁷ both when plated with (a) or without serum. Typical plaques of vinculin were also seen in these cells in IIF (b). Vinculin was isolated from chicken gizzard according to Feramisco and Burridge⁴¹. Antibodies were raised in rabbits and purified on antigen coupled to Sepharose 4B. Cells exposed to monensin on subcultivation showed only a diffuse actin-specific (c) or vinculin-specific (d) fluorescence in IIF. Such cells showed, however, typical arrays of both intermediate filaments (e), as shown with anti-vimentin antibodies⁴², and microtubules (f), as revealed with anti-tubulin antibodies¹⁷. When spread cells were exposed to monensin (1 μ M, 24 h), normal arrays of both microfilaments (g), phase-dense stress fibres (h) and vinculin plaques were still seen. For cytoskeletal antibodies the cells were fixed in -20 °C methanol for 5 min and stained for IIF as in Fig. 1. $\times 500$.

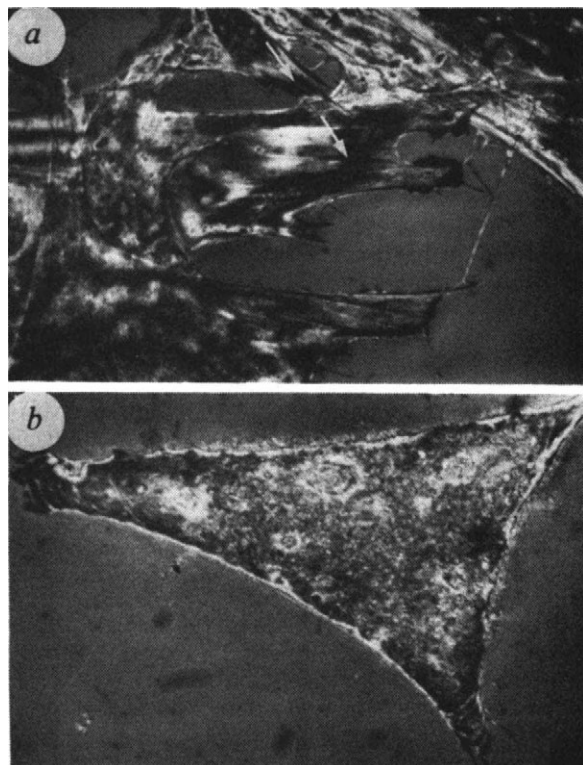


Fig. 5 Interference reflection microscopy¹⁷ of control human fibroblasts (a) and of cells exposed to monensin on subcultivation (b). Note the black areas of focal adhesion (arrows in a) and extensive grey areas of close adhesion in monensin exposed cells (b). $\times 900$.

adhesion sites, bundles of microfilaments and plaques of vinculin. This sequence of events corresponds to recent observations distinguishing a cell-independent and a cell-dependent phase in cell-to-substratum adhesion^{13,32-35}. We suggest that a prerequisite for cells to enter into the cell-dependent phase of spreading is the deposition of endogenous pericellular fibronectin and subsequent formation of focal adhesion sites and the microfilament-vinculin system which, in turn, are needed to maintain an isometric contraction in cells³⁶.

Interestingly, human embryonal fibroblasts which were allowed to spread in the presence of monensin, could not acquire the matrix-type of fibronectin even from the culture medium³⁷. This might be due to the lack of receptors for exogenous fibronectin in monensin-treated cells, as trypsinized cells are also apparently unable to retain exogenously added fibronectin³⁸. A recent study by Atherton and Hynes³⁹ using monoclonal antibodies has revealed differences in plasma and cellular fibronectins which may be of importance in their apparently different roles in cell adhesion, as suggested by our results.

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Evidence for contractile flexing of the gliding bacterium *Flexibacter* FS-1

Robert P. Burchard

Department of Biological Sciences, University of Maryland, Baltimore County, Catonsville, Maryland 21228, USA

Flexing movements have been described in a variety of gliding bacteria and cyanobacteria (blue-green algae)¹⁻⁷. Flexing of trichomes (chains of cells) of the genera *Oscillatoria* and *Beggiatoa* was attributed⁸ to a "certain degree of elasticity"; it was concluded that there is no spontaneous flexibility. In contrast, observations of active flexing of free ends of otherwise immobilized *Oscillatoria* trichomes suggested⁹ that active contractions are involved. It has been proposed that flexing and gliding motility are powered by the same mechanism¹⁰. No organelles have been demonstrated irrefutably to be responsible for these movements, although several mechanistic hypotheses have been proposed¹¹. I demonstrate here that flexing movements by the bacterium *Flexibacter* FS-1 require no more than one point of attachment by the cell to its substratum, which suggests that flexing may result from a unilateral, longitudinal contraction. As the same motility mechanism is thought to operate for both flexing and gliding, the latter may be powered by a contractile apparatus, at least in this species.

I and others⁶ have observed that flexing, like gliding, requires contact with a surface, such as another cell, a glass slide or an agar gel; in suspension such cells do not move actively. Gliding motility itself could account for flexing: for example, if the two polar regions of a cell or chain of cells were attached to a surface and were gliding in an uncoordinated fashion so as to be moving towards one another, the central portion of the cell(s) would, if not firmly attached to the surface, bow outwards and thus appear to flex. Reversal of the flexion could be explained by reversal of the direction of gliding at both ends. Uncoordinated gliding has been described in trichomes of the cyanobacterium *Oscillatoria*^{12,13}.

I have investigated this phenomenon by using a simple experimental design. *Flexibacter* FS-1, which grows as long filaments with widely spaced septa^{6,14}, was cultured in YE/2 medium¹⁰ at ambient temperature or at 30°C with little or no agitation. Cultures at absorbance (A_{540}) = 0.1, containing filaments $\leq 200 \mu\text{m}$ long (capable of active flexing in a wet mount



Fig. 1 Flexing *Flexibacter* FS-1 filaments attached to a glass wool fibre. Flexion points are indicated by arrows. Scale bar, 10 μm .

preparation), were diluted in YE/2 to $\sim 10^4$ filaments ml^{-1} and loaded into a 1 cm deep chamber mounted on a glass slide. A small tuft of glass wool fibres was submerged in the suspension. The chamber was then covered with a coverslip, and vacuum grease used to prevent leakage. The chamber was inverted and mounted on the stage of an inverted, phase-contrast microscope equipped with a $\times 10$ Zeiss achromat objective (5 mm working distance). It was thus possible to observe individual filaments which readily but reversibly attached at or near one pole (or by their lateral surfaces) to a glass wool fibre and made no contact with any other surface.

Some of these multicellular filaments were observed to flex and relax repeatedly (Figs 1, 2). Flexing assumed various forms, including localized bending and undulating of the filaments. Flexions could be observed to move along a filament towards the unattached end. This end sometimes abruptly flexed so as

to form a distinct hook and then very rapidly straightened out, as though the hook were spring-loaded or under some tension. As soon as the filaments detached from the glass wool, flexing ceased. The movements observed were active, as addition of dinitrophenol (5×10^{-4} M final concentration) inhibited flexing, although attachment appeared to have been unaffected. This agent also inhibited flexing in wet mounts and is known to inhibit gliding motility of *Flexibacter* and other gliding bacteria¹¹.

To ensure further that the observed flexing is an active process rather than one resulting from turbulence in the chamber, I examined the behaviour of strain Ng 7, a UV induced, non-gliding mutant of FS-1. The filaments ($\leq 40 \mu\text{m}$ long) of this mutant also attached to the glass wool fibres but did not flex in the conditions used nor in wet mounts.

This active flexing by multicellular filaments attached via one pole can most readily be explained by a contractile mechanism which is longitudinally arrayed and unilateral. Similarly, flexional and swinging movements by the free ends of aggregated *Oscillatoria* trichomes^{9,15} may also result from contractions. If gliding motility and flexing are indeed powered by the same mechanism, then gliding presumably also involves a contractile mechanism in *Flexibacter* FS-1.

Other theories to account for gliding motility have been proposed. The rotary assembly model¹⁶ is attractive as it provides a unified theory of prokaryotic motility; both flagellated^{17,18} and gliding bacteria would be driven by rotary motors. However, it is not clear how the spinning of rotary assemblies could explain the flexing movements described here, especially as flexing was observed in filaments with one end wrapped around a glass wool fibril and in those attached by one point on their lateral surface (Fig. 3). Similarly, 'make and break' interactions within the envelope¹⁹, the elaboration of surfactant gradients^{20,21}, directed production of extracellular slime²², and an electrokinetic mechanism²³ cannot account for the observed flexing.

Contractions have been described in a mycoplasma which also demonstrates a form of surface translocation called gliding²⁴. However, I am unaware of any previous definitive reports of contractile phenomena among enveloped prokaryotes.

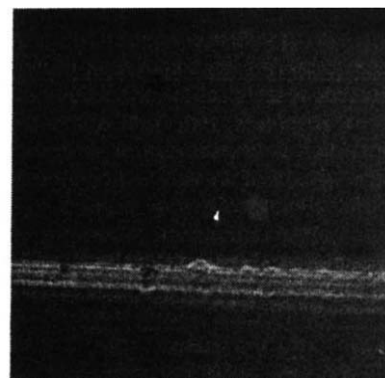
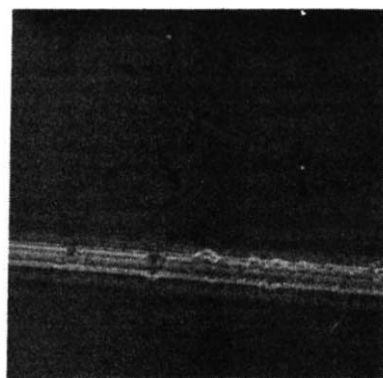
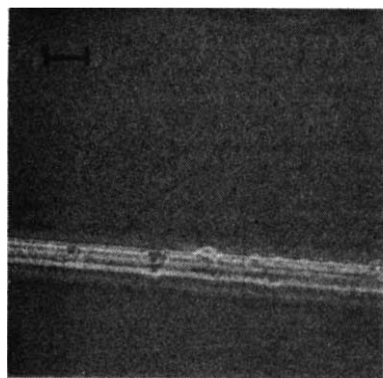
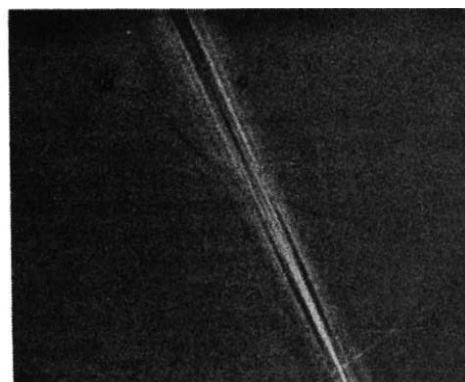
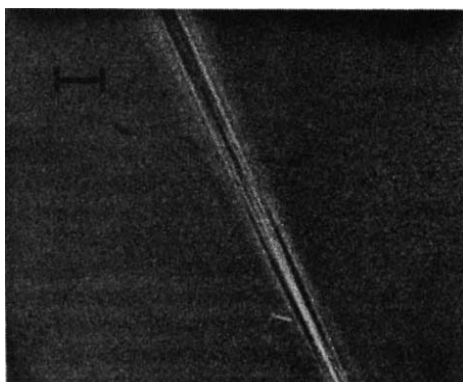


Fig. 2 A *Flexibacter* filament before and during a flexion (arrow). Scale bar, 10 μm .

Fig. 3 A flexing *Flexibacter* filament attached to a glass wool fibril along its lateral surface. Scale bar, 10 μm .



There is no known structural basis for contractility in *Flexibacter*. Attempts to isolate eukaryotic-like actin from this bacterium have been unsuccessful (R.P.B. and S. Mockrin, unpublished results). Organelles proposed to have contractile or bending properties and to be mechanistically involved in gliding have been described in *Myxococcus*²⁵ and in *Oscillatoria*²⁶. A contractile mechanism may, in fact, account for all of the forms of movement demonstrated by gliding bacteria²⁷.

A puzzling question which arises from these observations is why any point of attachment to a substratum should be required for flexing if this movement results from a contractile event. It may be that contact of the cell envelope with a surface is required to initiate such contractions.

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X-ray scattering by myosin S-1: implications for the steric blocking model of muscle control

Robert Mendelson

Department of Biochemistry and Biophysics, and Cardiovascular Research Institute, University of California, San Francisco, California 94143, USA

Steric blocking and unblocking^{1–3} of the myosin interaction sites in muscle thin filaments by tropomyosin provides a simple yet elegant method of regulating contraction in skeletal muscle. Recently, this model was challenged⁴ by the finding that tropomyosin is located on the opposite side of the thin filament helix axis from where Moore *et al.* reported that the myosin cross-bridge, S-1, bound⁵. However, new three-dimensional image reconstructions of electron micrographs of thin filaments decorated with S-1 were obtained by Taylor and Amos⁶, who proposed that the prior assignment of the S-1 binding site of Moore *et al.*⁵ was incorrect, and that consequently tropomyosin and S-1 are located on the same side of the actin helix. These past difficulties and the current lack of agreement⁷ on the location of the S-1 binding site suggested that another technique would be useful. We report here that the shape of S-1, derived from reconstructions using the Taylor and Amos actomyosin interpretation, gives a better fit to the X-ray scattering pattern observed from S-1 in solution than alternative interpretations.

The two assignments of S-1 position in the three-dimensional reconstructions are illustrated in Fig. 1. The original assignment by Moore *et al.* was based on the supposition that a radial

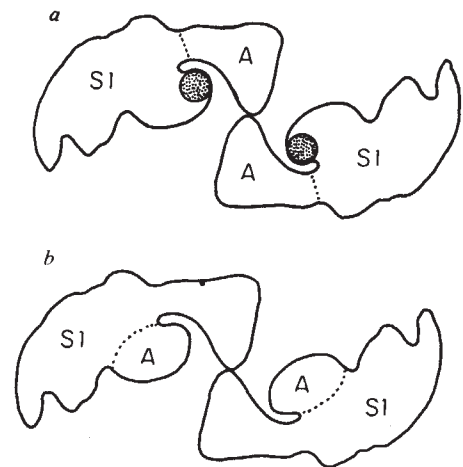


Fig. 1 A projected view (from the three-dimensional reconstructions of ref. 5) looking down the thin filament axis towards the Z-line with the S-1 position drawn according to the interpretation of: *a*, Taylor and Amos, and *b*, Moore *et al.* In the steric blocking model the tropomyosin (shaded in *a*) is thought to unblock the S-1 attachment site as the amount of Ca^{2+} binding to troponin (located at every seventh actin monomer) increases. The position of tropomyosin shown is approximately that given by ref. 4 for the rigor or activated state. If the interpretation of Taylor and Amos is correct, the S-1 binding site is nearer tropomyosin, and the shape of S-1 is more compact than in the Moore *et al.* interpretation. The structure of Moore *et al.* is similar to that shown, except that a significant amount of the mass labelled A is missing at low radii.

expansion of the acto-S-1 helix had occurred, as had been found in some isolated F-actin reconstructions. Using minimal dose electron microscopy with improved specimen preparation, Taylor and Amos found additional mass near the helix axis which they assigned to actin. They argued that the filaments of Moore *et al.* had not expanded radially and the mass previously assigned to actin was actually part of S-1. Although their assignment agrees well with the known mean radius of actin, the Moore *et al.* interpretation is difficult to refute with certainty because of the inherent ambiguity in defining a boundary between two complexed molecules whose individual structures are not highly determined. As the S-1 derived from the two proposed locations of the S-1 binding sites have very different shapes, we tested these contrasting models by X-ray scattering from isolated S-1 in solution.

Previous X-ray scattering results⁸ showed that S-1 shapes derived from three-dimensional helical electron micrograph reconstructions were capable of giving a better fit to scattering data than many simple models that might resemble S-1. Recent high resolution reconstructions, in addition to raising the question of S-1 position, suggest that the shape of S-1 is significantly altered by the presence of the 18,000 molecular weight (M_r) light chain⁹. Previously reported scattering data were from S-1 that contained only a fraction of the 18,000- M_r light chain. Thus, scattering data were accumulated on S-1 both with and without this light chain. The fractional change in the radius of gyration (R_g) was 0.01 ± 0.005 for S-1 with and without the light chain (five double preparations^{10,11}, seven trials), and the scattering patterns (Fig. 2) and chord distributions were almost identical, indicating that the 18,000- M_r light chain is not a significant determinant of S-1 shape. Thus, the present results validate the results of previous modelling studies⁸ for S-1s that have varying amounts of light chain.

If the addition of light chain causes an isotropic increase in size, it would be expected that $\Delta R_g/R_g = 1/3 (\Delta M_r/M_r) = 0.05$, when using the molecular weights of Margossian *et al.*¹². Thus, S-1 with 18,000- M_r light chain is slightly more symmetrical than S-1 without it. The disagreement of the present result with those computed from reconstructions of decoration of actin by scallop S-1 ($\Delta R_g/R_g = 0.15$)⁹ may be only apparent; our studies

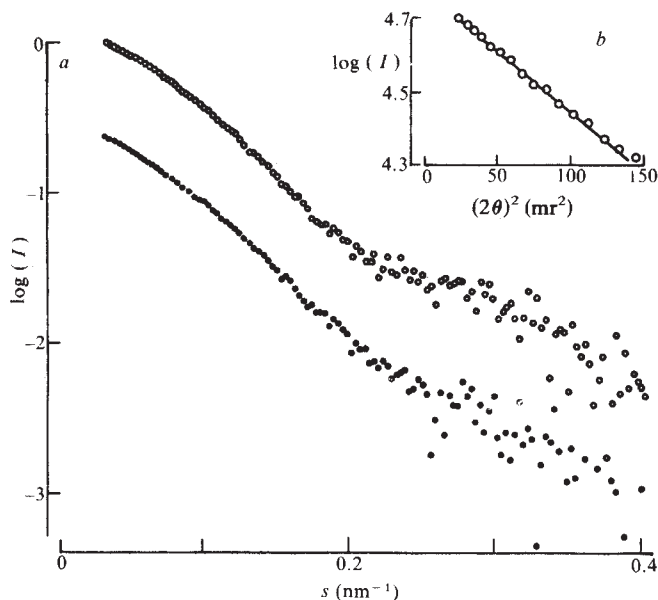


Fig. 2 *a*, Typical scattering patterns from S-1 with (●) and without (○) the 18,000- M_r light chain. Rabbit S-1 with this light chain was prepared and purified as described in ref. 10 except that the S-1 yield was kept below 20% so as to minimize heavy chain degradation. S-1 without the light chain was prepared as in ref. 11 except that purification was by S-200 gel filtration, which did not separate S-1 isoenzymes. The buffer contained 150 mM KCl; 20 mM tri[hydroxymethyl]methylaminoethane sulphonic acid; 0.1 mM NaN_3 ; 1 mM dithiothreitol, pH 7 at $T = 4^\circ\text{C}$; $[\text{S-1}] = 7 \text{ mg ml}^{-1}$. *b*, A Guinier plot from the lowest angle portion of the scattering curve, where I is the scattering intensity, and 2θ is the scattering angle. Guinier plots and chord distributions appeared virtually identical with and without light chain. Data were accumulated from $s = 1/38 \text{ nm}^{-1}$ to about $s = 1/2.5 \text{ nm}^{-1}$ (where $s = 2 \sin \theta / \lambda$) using a Kratky camera with a position-sensitive proportional counter. The sample-to-registration-plane distance was 214 mm. The range of S-1 concentrations was 7–32 mg ml^{-1} . $\text{mr} = \text{milliradians}$.

were performed on rabbit skeletal S-1, which shows a less barbed structure than scallop S-1 in acto-S-1 micrographs¹³. In addition, the R_g and chord distribution (not shown) of scallop S-1 without 18,000- M_r light chain were in good agreement with the data; thus, an intact, flexible, light chain could be oriented most frequently along the surface of the S-1 heavy chain to

give on average a compact S-1 structure in solution, while the electron microscopy stain could cause it to be visualized only in an extended orientation which would make the overall structure appear more elongate.

Comparison of the S-1 shapes derived from the two different interpretations is shown in Table 1 and Fig. 3. The radius of gyration (R_g), which is derived from the lowest resolution portion (smallest angle) of the scattering curve, most directly reflects the overall shape of the protein. As shown in Table 1, the R_g 's showed a consistent preference for the Taylor and Amos interpretation in the micrographs of both Moore *et al.* and Taylor and Amos. The interpretations of the Wakabayashi and Toyoshima⁷ reconstruction in terms of the two aforementioned S-1 positions are not fully consistent with the findings of other workers; their assignment of the Moore *et al.* actin position has a lower radius than that of their Taylor and Amos actin position. Further, their proposed actin structure, in both interpretations, has a smaller volume, relative to S-1, than actin in the reconstruction of Taylor and Amos. However, even this study favours a Taylor and Amos type of S-1 binding position. The mean and the maximum chord can be extracted from the chord distributions¹⁴ and directly compared with these parameters from the reconstructions. As the mean chord (and R_g) is an average that depends on the entire structure of the molecule, it is a better parameter for experimental comparison than the maximum chord. The mean chord and relative goodness of fit factors to the chord distributions obtained in this study also favour the Taylor and Amos interpretation, although the maximum chord was inconclusive.

The choice of different micrograph contour levels had little effect on the overall shape of S-1; the difference in R_g using two plausible extreme contours of the model in ref. 5 (provided by K. Taylor) was only 0.05 nm when the contours were scaled to give the correct S-1 volume. In the previous X-ray scattering study⁸, a good fit was obtained using a different reconstruction (J. Seymour and E. J. O'Brien, unpublished). Although the actin and tropomyosin were not fully resolved from S-1 in this reconstruction, their subtraction was consistent with that of Taylor and Amos (J. Seymour, personal communication). Indeed, alignment of this reconstruction with that of Taylor and Amos indicated that the shape and location of S-1 were very similar to those of Taylor and Amos. The results of X-ray scattering by S-1 in solution substantiate the shape of S-1 derived when it is attached to actin in the manner proposed by Taylor and Amos rather than in the position of Moore *et al.* Although we cannot formally exclude the possibility that the

Table 1 Structural parameters of S-1 from electron microscopy and X-ray scattering

Author and S-1 position	R_g (nm)	Mean chord (nm)	Max chord (nm)	$R(\text{Moore})/R(\text{Taylor})^*$
Moore <i>et al.</i> (x, y, z)				
Moore <i>et al.</i> S-1	3.83	4.80	15.1	11
Taylor & Amos S-1	3.38	4.35	12.8	
Taylor & Amos (x, y)				
Moore <i>et al.</i> S-1	3.93	4.97	13.5	7
Taylor & Amos S-1	3.18	4.06	12.8	
Wakabayashi & Toyoshima (x, y)				
Moore <i>et al.</i> S-1	3.51	4.46	12.8	1.3
Taylor & Amos S-1	3.14	4.07	11.5	
Wakabayashi & Toyoshima (x, y, z)				
Moore <i>et al.</i> S-1	3.49	4.43	12.5	1.5
Taylor & Amos S-1	3.21	4.14	11.0	
Experimental†				
No LC2 ($M_r = 110 \text{ K}$)	3.27 (0.1)	4.25 (0.15)	12 (1)	
Part LC2 ($M_r = 122 \text{ K}$)	3.28 (0.06)	4.25 (0.15)	12 (1)	
Full LC2 ($M_r = 130 \text{ K}$)	3.3 (0.1)	4.25 (0.15)	12 (1)	

Letters in parentheses denote the method of scaling suggested by each author. Wakabayashi and Toyoshima's maps were also scaled on x, y, z to illustrate the effect of a different scaling method on the parameters. K, molecular weight 1,000.

$$^* R = \frac{\{\sum_i [p_o(r_i) - p_c(r_i)]\}^2}{\{\sum_i p_o(r_i)\}^2}$$

where $p_o(r)$ is the observed and $p_c(r)$ is the calculated chord distribution function.

† All maps are to be compared with 'no LC2' except those of Moore *et al.*, which is to be compared with 'part LC2'. Any error in the findings that true molecular weight scales with LC2 content will improve the agreement with the Taylor and Amos interpretation.

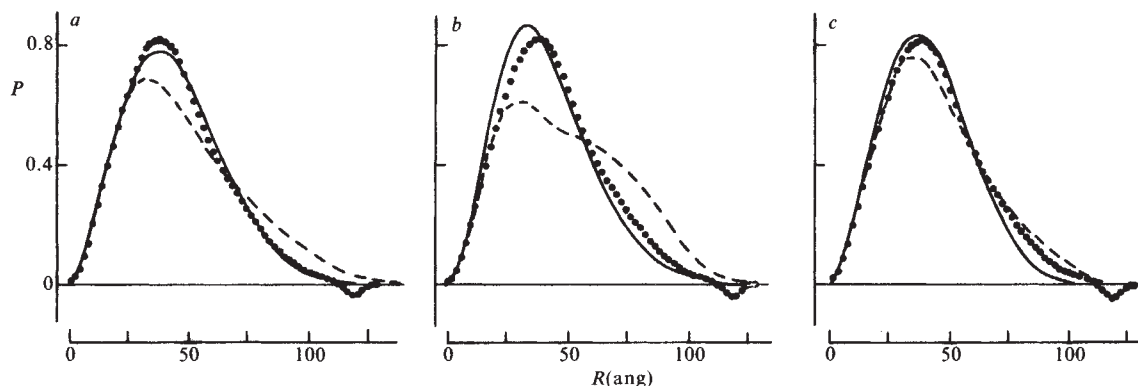


Fig. 3 A comparison of the S-1 experimental (●) chord distribution with the predictions from electron microscopy using the Taylor and Amos interpretation (solid lines) and the Moore *et al.* interpretation (dashed lines). The electron density maps were from: *a*, Moore *et al.*; *b*, Taylor and Amos; *c*, Wakabayashi and Toyoshima. The maps were furnished with the actin-S-1 interfaces delimited by their authors except in the case of the Moore *et al.* map, where the interface was delimited by L. Amos. The maps were scaled to the correct volume. This was done by scaling each *x* and *y* coordinate or each *x*, *y* and *z* (helix axis) coordinate as recommended by the author. Typically, the scaling changed these dimensions by only 10–20%. Tests showed that the method of scaling had only a small effect on the predicted chord distributions and on the overall molecular shape (see Table 1). Chord distributions were obtained by Fourier transforming the experimental and model scattering curves using the method of Glatter¹⁴.

S-1 solution could have a shape similar to that found by Taylor and Amos and yet makes a conformational change to the shape described by Moore on binding to actin⁷, such a large change seems improbable because the accompanying R_g change would be of an unprecedented magnitude. Thus, the present results are consistent with the notion that regulation of muscle contraction occurs by the steric blocking of the actin-S-1 interaction.

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Insulin stimulates tyrosine phosphorylation of the insulin receptor in a cell-free system

Masato Kasuga*, Yehiel Zick†, Diana L. Blithe‡, Marco Crettaz* & C. Ronald Kahn*

* Elliott P. Joslin Research Laboratory, Joslin Diabetes Center and Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts 02215, USA

† Diabetes Branch, National Institutes of Arthritis, Diabetes and Digestive and Kidney Diseases, and ‡ Developmental Endocrinology Branch, National Institutes of Child Health and Human Development, Bethesda, Maryland 20205, USA

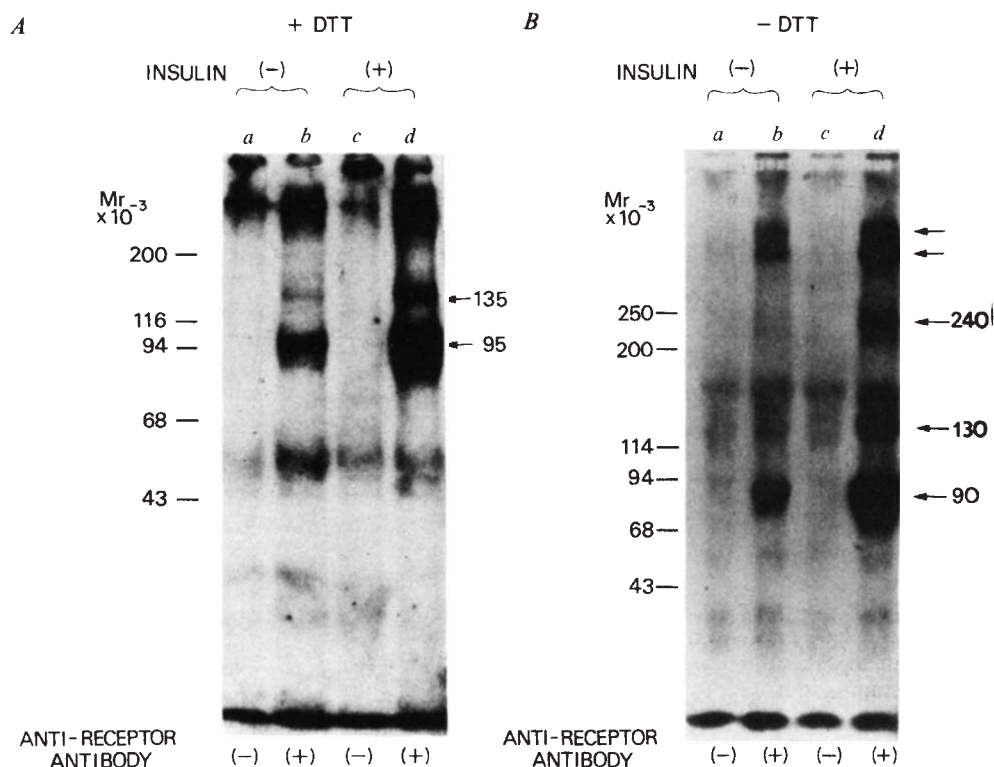
Insulin forms a complex with its receptors on the plasma membrane of cells and initiates a series of biochemical events which lead to the hormone's recognized effects^{1,2}. The exact mechanism that initiates these events is unknown. We previously reported that insulin stimulates the phosphorylation of the 95,000 molecular weight (M_r) (β) subunit of its own receptor in intact cells and proposed that this phosphorylation reaction could be a very early step in insulin action³. To clarify the molecular basis of this reaction, we have now investigated the phosphorylation of insulin receptor in a cell-free system. Using [γ -³²P]ATP in solubilized and partially purified receptor preparations from rat liver plasma membrane, we find that both the α and β subunits of the insulin receptor are phosphorylated. Furthermore, insulin stimulates the incorporation of ³²P into both receptor subunits in a specific and dose-dependent manner. Phosphoamino acid determination of the β subunit after insulin stimulation reveals only phosphotyrosine. These findings suggest that the elements required for phosphorylation are associated with the plasma membrane of the cell and that specific phosphorylation of the insulin receptor on tyrosine residues can be activated in a solubilized preparation.

Rat liver plasma membranes were prepared by the procedure of Neville⁴ and phosphorylation studied using [γ -³²P]ATP as described in Fig. 1 legend. Insulin receptors in this rat liver membrane fraction and solubilized insulin receptors prepared from these membranes have been well characterized^{5,6}. Further, as previously shown lectin chromatography allows a 20-fold purification with nearly 100% recovery of insulin receptors as determined by ¹²⁵I-insulin binding⁷. The insulin receptor subunits were identified by immunoprecipitation with antibodies against insulin receptor followed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography^{3,8}.

When the immunoprecipitates were analysed in SDS-PAGE in reducing conditions (100 mM dithiothreitol; DTT), one major band of M_r 95,000 and a minor band of M_r 135,000 were specifically phosphorylated in basal state (Fig. 1A, lane *b*). These 135,000- and 95,000- M_r phosphoproteins have been tentatively identified as the α and β subunits of insulin receptor, respectively, by their immunoprecipitation by anti-receptor antibody and by the fact that they migrate in the same position in SDS-PAGE as α and β subunits of insulin receptor labelled either biosynthetically or externally^{3,8}. After incubation of the solubilized fraction with insulin (10^{-6} M), the incorporation of ³²P into these two proteins was increased approximately fivefold as determined by scanning densitometry (Fig. 1A, lane *d*). A phosphoprotein band corresponding to M_r ~50,000 is also observed; this may represent phosphorylation of the heavy chain of IgG.

In the native insulin receptor, the receptor subunits are thought to be linked by disulphide bonds to form high molecular weight oligomers^{9,10}. When the same samples were analysed without reduction of disulphide bonds, five bands were observed (Fig. 1B, lane *b*). Two bands had molecular weights higher than 300,000. In addition, there were phosphoproteins of M_r s 240,000, 130,000 and 90,000 in basal state (Fig. 1B, lane *b*); these bands are identical to those found when the receptor

Fig. 1 Autoradiogram showing the incorporation of the ^{32}P from $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ into solubilized insulin receptor from rat liver plasma membrane. Rat liver plasma membranes were prepared by the procedure of Neville⁴. Protease inhibitors (2 mM phenylmethylsulphonyl fluoride; 1,000 trypsin inhibitor units per ml aprotinin) were added throughout the purification beginning with the first step of homogenization. The plasma membrane fraction was solubilized by 1% (v/v) Triton X-100 in 50 mM HEPES buffer, pH 7.6, containing protease inhibitors. The insulin receptors were further enriched by chromatography on a wheat germ agglutinin-agarose column⁷ after elution by 0.3 N *M*-acetyl glucosamine in 50 mM HEPES buffer, pH 7.6, containing 0.1% (v/v) Triton X-100. Aliquots of these eluted fractions (150–300 μg per tube) were incubated with or without insulin (10^{-6} M) overnight at 4 °C. A 1-h incubation with insulin at room temperature was also used. Essentially the same result was obtained in the two different incubation conditions. The aliquots were then assayed for the incorporation of ^{32}P from $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ at 4 °C in a final volume of 1,350 μl . For the experiment shown, the reaction mixture contained 6 mM MgCl_2 , 2 mM $\text{Mn}(\text{CH}_3\text{COO})_2$, 5 μM $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ ($\sim 30 \mu\text{Ci nmol}^{-1}$) in 30 mM HEPES buffer, pH 7.6. Similar results were obtained with 20 mM MgCl_2 and 1 mM MnCl_2 in a 100 mM HEPES buffer, pH 7.4. Assays were initiated by the addition of ATP. After 10 min incubation, NaF, sodium pyrophosphate, EDTA, ATP and Triton were added to get final concentration of 50, 10, 5 and 5 mM and 0.1% (v/v) respectively. The insulin receptor was quantitatively immunoprecipitated by serum containing antibody against insulin receptor (serum B-2, 1:200 dilution) or normal control serum⁸. After 2.5 h at 4 °C, 200 μl of Protein A (Pansorbin; 10% w/v) were added and the incubation continued for 1 h at 4 °C. The precipitates were then collected by centrifugation at 10,000g for 5 min at 4 °C and washed twice with 1% Triton and 0.1% SDS⁸. Immunoprecipitates were solubilized by boiling for 3 min in 2% SDS in 10 mM sodium phosphate, pH 7.0, with (A) or without (B) dithiothreitol (DTT). The labelled components were separated electrophoretically in 5 or 7.5% polyacrylamide slab gels in the presence of 0.1% SDS as described by Laemmli³². After electrophoresis, the slab gels were stained, destained, dried and autoradiographed for 3–7 days as previously described⁸. Subunit molecular weights were calculated by using as standards (M_r): filamin (250,000), myosin (200,000), β -galactosidase (116,000), phosphorylase *b* (94,000), bovine serum albumin (68,000) and ovalbumin (43,000).



is labelled by ^{35}S -methionine or ^3H -sugars and analysed without reduction¹¹. Using two-dimensional (non-reducing/reducing) gel electrophoresis, we have previously shown that the two highest molecular weight bands are composed of α and β subunits, and three other bands are an α - α dimer and 'free' α and β subunits respectively¹¹. After incubating with insulin (10^{-6} M), the phosphorylation of all five bands was increased approximately five- to six-fold.

To evaluate further the mechanism of phosphorylation of insulin receptors, the solubilized fractions were incubated with several concentrations of insulin (10^{-10} to $\sim 10^{-6}$ M), desoctapeptide (DOP) insulin and multiplication-stimulating activity (MSA), one of the insulin-like growth factors, and the phosphorylation was carried out as described above. Physiological concentrations of insulin (3×10^{-10} M) increased the phosphorylation of both subunits approximately threefold (Table 1). Stimulation was maximal with insulin concentrations of 10^{-8} to $\sim 10^{-6}$ M (Table 1; Fig. 1). DOP insulin, which has $\leq 1\%$ of the potency of porcine insulin in displacing bound ^{125}I -insulin from rat liver membrane insulin receptor and stimulating glucose metabolism⁶, stimulated phosphorylation only minimally. MSA (10^{-7} M) was also far less potent than the same concentration of porcine insulin (Table 1), as expected from previous studies of the affinity of MSA for the insulin receptor in rat liver plasma membrane^{12,13}. Thus, the 135,000- and 95,000- M_r bands were phosphorylated in dose-dependent fashion and specificity consistent with the affinity of the insulin receptor for insulin analogues.

To determine the phosphoamino acids of the β subunit of the insulin receptor, this ^{32}P -labelled phosphoprotein was cut from the gel and partially hydrolysed in 6 M HCl for 2 h at 110 °C. The resulting hydrolysates were separated by two-dimensional electrophoresis (pH 2.0 and pH 3.5) in the

presence of unlabelled phosphoserine, phosphothreonine and phosphotyrosine, and the autoradiograms of the separated phosphoamino acids were compared with the ninhydrin-stained standards. Only trace amounts of phosphotyrosine could be detected in the basal state (data not shown). After insulin stimulation (10^{-7} M), there was a dramatic increase in the amount of phosphotyrosine (Fig. 2). Because of the low radioactivity, phosphoamino acid in α subunit could not be identified.

There is considerable evidence that phosphorylation and dephosphorylation may have various roles in insulin action^{14–20}. Recently, we found that insulin stimulates the phosphorylation

Table 1 Effects of insulin and insulin analogues on ^{32}P incorporation into solubilized insulin receptor subunits from rat liver plasma membrane

Addition	Concentration (M)	³² P incorporated into insulin receptor subunits (arbitrary units)	
		M _r 135,000	M _r 95,000
Expt 1			
None	—	3.7	20.2
Porcine insulin	3 × 10 ⁻¹⁰	12.9	64.0
Porcine insulin	1 × 10 ⁻⁷	21.5	116.0
Desoctapeptide insulin	1 × 10 ⁻⁷	4.1	27.2
Expt 2			
None	—	<0.5	5.2
Porcine insulin	1 × 10 ⁻⁷	4.0	38.2
Multiplication- stimulating activity	1 × 10 ⁻⁷	<0.5	6.6

Experiments 1 and 2 were performed with solubilized fractions derived from different membrane preparations. The studies were carried out as described in Fig. 1 legend. The autoradiograms were scanned in a Joyce-Loebl microdensitometer and the peak areas corresponding to the 135,000- and 95,000- M_r bands were calculated and expressed in arbitrary units.

of the β subunit of the insulin receptor in intact cells, indicating that this may be an early step in insulin action. This study demonstrates that the same phenomenon can be reproduced in cell-free systems and suggests several interesting points. First, although we are unable to determine the source of the phosphate used to phosphorylate the 95,000- M_r protein in an intact cell, in the broken cell system this protein clearly accepts the phosphate from γ -labelled ATP. Second, the results suggest that an endogenous protein kinase(s) is(are) involved in this reaction and that this endogenous protein kinase activity exists in the plasma membrane fraction and after affinity chromatography on wheat germ agglutinin-agarose. Furthermore, insulin stimulates the incorporation of ^{32}P into the β subunit of insulin receptor at tyrosine residues only. Phosphorylation of tyrosine is rare in normal cells²¹, but specific tyrosine phosphorylation of other proteins has been observed after cellular transformation by RNA tumour viruses²¹⁻²⁵ or stimulation of cell growth by epidermal growth factor (EGF)^{26,27}, platelet-derived growth factor²⁸ and a human transforming growth factor²⁹. Insulin is also a strong growth promoter.

Although phosphorylation has been demonstrated in both the intact and broken cell, there are some differences between them. First, the dose-response curve of phosphorylation is shifted to the left, that is, it is more sensitive, in a cell-free system than in the intact cell. This may depend on several factors, including the conditions of assay and the source of membranes. Second, in the intact cell only the β subunit is phosphorylated, whereas in the cell-free system, both subunits of the insulin receptor are phosphorylated. This suggests the α subunit can also be a substrate for endogenous protein kinases, although the amount of phosphorylation of the α subunit is usually 15% or less than that of the β subunit. The reason we cannot detect the phosphorylation of α subunit in intact cells may be related to the low level of phosphorylation, to the topography of insulin receptor subunits in intact cells, to differences in the level of endogenous phosphorylation, or to differences between these liver membranes derived from normal rats and cultured tumour cells. Third, we have found that in the intact cell, the content of both phosphoserine and phosphotyrosine in the β subunit of the insulin receptor is increased after insulin stimulation³⁰, although in the intact cell the absolute amount of phosphotyrosine is very small compared with phosphoserine. In the broken cell system, however, only phosphotyrosine is found. These results suggest that the tyrosine

phosphorylation of the β subunit may be the initial reaction which occurs on insulin binding and that phosphorylation of serine requires other components absent in cell-free systems. Similar differences of phosphoamino acid composition between intact cells and cell-free systems have been observed in the phosphorylation of RNA tumour virus oncogene products^{21,23-25}, and the receptor for EGF^{26,27}.

Our results provide the first demonstration, in a cell-free system of a covalent modification of the solubilized insulin receptor as a consequence of insulin-receptor complex formation. The mechanism by which insulin stimulates the incorporation of ^{32}P from $[\gamma\text{-}^{32}P]\text{ATP}$ into its own receptor at tyrosine residue is not known. This system does, however, provide the opportunity to investigate whether the insulin receptor is a tyrosine protein kinase, as suggested for the EGF receptor³¹, or a regulator of phosphoprotein phosphatase.

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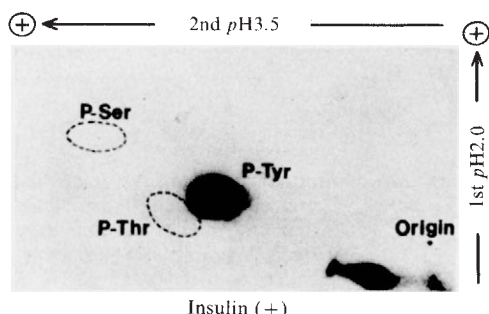


Fig. 2 Identification of phosphoamino acids in the 95,000- M_r phosphoprotein. The phosphoamino acids were analysed by a modification of the method of Hunter and Sefton²¹. The phosphorylation of the 95,000- M_r protein (β subunit of insulin receptor) was stimulated by insulin (10^{-7} M) as shown in Fig. 1. The 95,000- M_r bands, localized by autoradiography, were excised from the gel and the proteins eluted by electrophoresis at 150 V for 20 h into a dialysis bag containing 10 mM sodium phosphate buffer (pH 7.0) and 0.05% SDS. The samples were dialysed against 10 mM NH_4HCO_3 , lyophilized, extracted by ethanol/ether (1:1 v/v) and then subjected to acid hydrolysis in 6 M HCl for 2 h at 110 °C. After lyophilization, the hydrolysates were spotted on Whatman 3MM paper. Electrophoresis was performed at pH 2.0 (formic acid/acetic acid/ H_2O , 81:25:879) and 550 V for 60 min at 12 °C in the first dimension, and at pH 3.5 (pyridine/acetic acid/ H_2O , 1:10:189) and 1,000 V for 105 min in the second dimension. Samples of authentic phosphoserine (Sigma), phosphothreonine (Sigma) and phosphotyrosine (a gift from Dr T. Hunter, Salk Institute) were added to all radioactive samples analysed. The standards were located by ninhydrin and are delineated by the broken lines. The radioactive material was located by autoradiography.

Insertion of diphtheria toxin into and across membranes: role of phosphoinositide asymmetry

James J. Donovan^{*†}, Melvin I. Simon^{*} & Mauricio Montal^{*†}

Departments of ^{*}Biology and [†]Physics, University of California, San Diego, La Jolla, California 92093, USA

Diphtheria toxin (DT), a 63,000-molecular weight soluble protein, is toxic to most mammalian cells. The mechanism of intoxication involves a step in which one part of the molecule inserts into a membrane, facilitating the transport of the enzymatic fragment of the protein into the cytoplasm^{1,2}. This event requires an acidic environment and seems to occur at the membrane of an endocytic vesicle^{3,4}. Different cell lines and species differ in their sensitivities to DT—this is due at least in part to differences in the number of DT surface receptors on the cells⁵, but may also arise from differences in the membrane transport step. It is generally recognized that protein-lipid

interactions are of critical importance in membrane transport phenomena (see ref. 6). Thus, it is of interest to determine whether the interaction of DT with membranes is modulated by their composition. We have shown previously that DT interacts with planar lipid bilayers at acidic pH to produce voltage-dependent channels⁷. We report here that this interaction of DT with bilayers depends on membrane phospholipid composition; the interaction is optimal when inositides are present within the membrane, and the inositides are required on the side of the membrane opposite to that in which DT is introduced.

We investigated the effect of membrane composition on the interaction of DT with lipid bilayer membranes. Figure 1a illustrates the time course of the membrane current at a constant applied voltage of -40 mV recorded from two different membranes. The upper trace corresponds to a phosphatidylethanolamine (PE) membrane and the lower to an asolectin (AL, soybean phospholipids) membrane. AL is a mixture of phospholipids, the major components of which are PE, phosphatidylcholine (PC), phosphatidylinositol (PI), and phosphatidylserine (PS), and contains a small amount of α -tocopherol. The conductance of each unmodified membrane was low, as indicated by the small current in response to a voltage step. On addition of DT to the aqueous compartment, the conductance of the asolectin membrane increased due to the formation of ionic channels, whereas the current through the PE membrane remained almost unchanged. Channels do form in PE membranes if sufficient time is allowed, and if higher DT concentrations are used, but PE membranes are consistently much less sensitive to DT.

Alving *et al.*⁸ have shown that the binding of DT to phospholipid vesicles at acidic pH is correlated with the membrane surface charge. This suggests that the difference between asolectin ($\sim 20\%$ negative lipids) and PE membranes might be due to differential binding resulting from differences in surface charge or lipid composition. To evaluate this hypothesis, we tested a variety of lipids in our bilayer system. The data are summarized in Table 1, where the average rate of conductance increase for several lipids is shown normalized to the rate of increase for asolectin at a DT concentration of 100 ng ml^{-1} . Membranes consisting of the neutral lipids PC and PE are relatively insensitive to DT, but the same is true for the negative phospholipid, PS and the negative lipid oleoyl phosphate (OP) at surface charge concentrations comparable to asolectin. Similarly, glycolipids or gangliosides do not sensitize the membrane to DT. Diposphatidylglycerol (DPG, cardiolipin) seems to confer some slight sensitivity to DT, but greater effects result from the presence of inositides in the membrane. As phosphatidylinositol is a major component of asolectin ($\sim 14\%$)^{9,10}, we believe that the PI in asolectin membranes is responsible for their sensitivity to DT. The fact that the PI-PC mixture is less DT-sensitive than asolectin could be due to the phytanoic acid chains on the PC because the acyl chain composition of the lipids affects DT binding⁸. Indeed, membranes formed with purified soybean PC, PE and PI exhibited DT-induced conductances comparable to asolectin, whereas substitution of PS for PI in this mixture rendered the membranes insensitive to DT (see Table 1). Channels do form in inositide-free membranes, but at a slower rate.

The rate of channel formation induced by DT is enhanced by the presence of a pH gradient. Similar results have been reported by Kagan *et al.*¹¹ who showed that a mutant form of DT, CRM 45, forms channels, and that the rate of conductance increase is greater in the presence of a pH gradient. For DT, we observed that in the presence of a 2-unit pH gradient (4.9–6.9, acid on the DT-containing side), the rate of channel formation was enhanced ~ 50 -fold, regardless of the composition of the membrane. Thus, the relative conductances are unchanged by the pH gradient. To separate clearly the effects of pH and membrane composition, all the experiments reported here were performed with both sides of the membrane at pH 4.85.

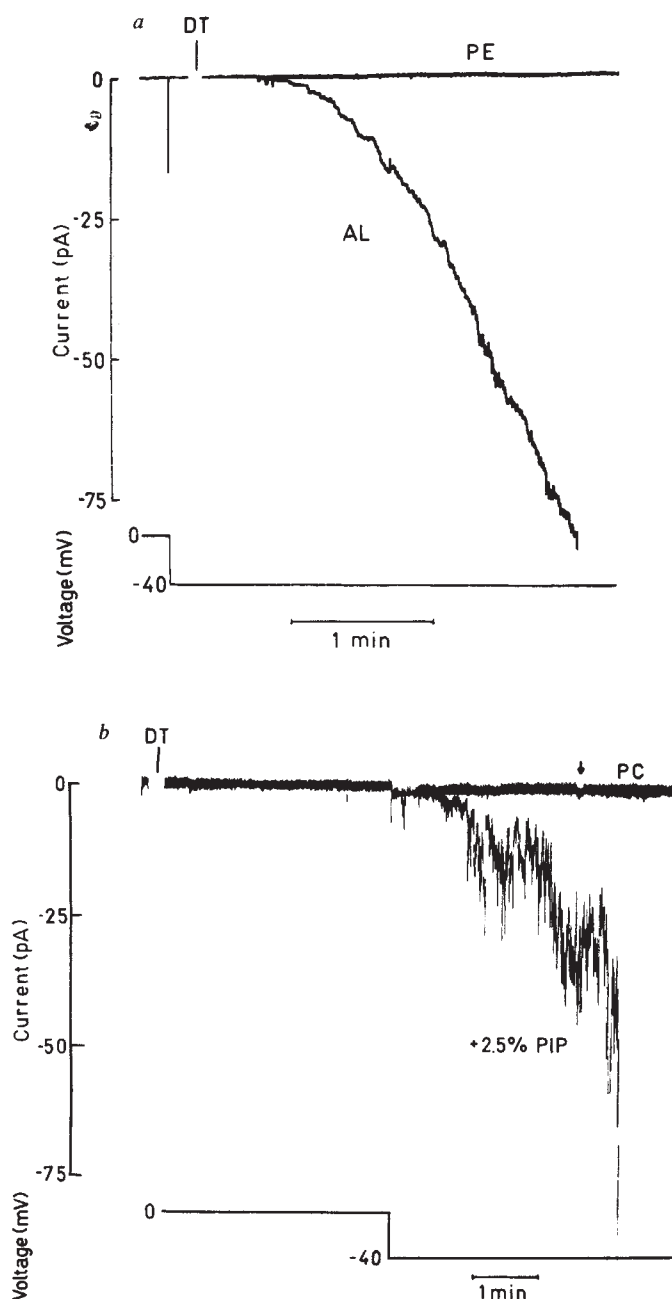


Fig. 1 Membrane currents at a constant applied voltage through symmetrical membranes treated with DT. Planar lipid bilayers were formed from monolayers as described elsewhere²³ across a $200\text{-}\mu\text{m}$ hole in a $12\text{-}\mu\text{m}$ -thick Teflon partition separating two 4-ml capacity Teflon compartments. The hole was treated with a 1% solution of squalene in pentane. Voltage was applied, and current measured using Ag/AgCl electrodes with an Analog Devices AD515 amplifier having a $1\text{-G}\Omega$ feedback resistor. The DT-containing compartment is defined as being at zero potential, and positive current is the flow of positive ions into this compartment. *a* Shows the current through two membranes before and after the addition of DT. The upper trace corresponds to a PE membrane, and the lower trace to an AL (soybean lipids purified by the method of Labarca *et al.*²⁴) membrane. *b* Shows two current traces: the upper trace is from a membrane consisting of 100% PC (diphytanoyl phosphatidylcholine, DPPC) and exhibits a low conductance during which a single channel of DT opens and closes (arrow). The lower trace is from a membrane containing 97.5% PC and 2.5% PIP. Because of the low solubility of PIP in alkane solvents, the lipid solution was sonicated briefly before spreading the monolayers. All experiments shown were done at room temperature using $0.2 \text{ M NaCl}/20 \text{ mM citrate buffer}$, pH 4.85. The DT concentration was 100 ng ml^{-1} . DT (Connaught) was purified by DE52 chromatography²⁵.

Another parameter which affects the potency of DT is nicking of the toxin. DT was nicked according to Drazin *et al.*¹². The conductance induced by nicked toxin was increased relative to un-nicked toxin by an order of magnitude, and showed less variability.

Most interesting among the lipids studied here is phosphatidylinositol-4-monophosphate (PIP)⁸. This lipid (PI with an extra phosphate on the inositol ring) has a double negative charge at the pH of these experiments. It is most effective at sensitizing the membrane to DT, which seems to be due to the inositol or phosphoinositol moiety and not the charge, as diphosphoglycerate is also doubly charged, but is much less effective. Figure 1b illustrates the ability of PIP to sensitize a membrane to DT. The two superimposed current traces are for two different membranes, one of 100% PC and another of 97.5% PC with only 2.5% PIP. The addition of this small amount of PIP caused the current to increase dramatically in the presence of DT, even though the surface charge density was about one-quarter that for an asolectin membrane. Such current increases were not seen in the absence of DT.

The simplest explanation for these data is that DT binds to inositides, especially PIP, on the bilayer surface^{8,13}. To test this notion, we prepared asymmetric lipid bilayers containing PIP exclusively in one monolayer. These membranes were made with lipid mixtures designed to have the same surface charge density in both monolayers. All other conditions, including DT concentration and pH, were also symmetric. Two superimposed traces of the current through asymmetric bilayers after the addition of DT are shown in Fig. 2a. When DT was added to the chamber in contact with the PIP face of the membrane, the membrane current was unaffected, except for some incidental current drift. In contrast, when DT was added to the PS side, the current increased dramatically after a significant lag time. Thus, although PIP does bind DT⁸, it does not mediate channel formation primarily through DT binding, but has a more subtle role. We have shown previously⁷ that the DT insertion and channel formation steps are distinct from the binding step. Our results indicate that phosphoinositides exert their effect not so much on the binding of DT but rather on either the insertion or channel formation step, and that they mediate this effect from the side opposite to that on which the DT is bound.

Table 1 Relative conductances induced by DT in symmetrical lipid bilayers of different composition

Membrane composition	Relative conductance*
Asolectin	1.0 ± 0.2 (10)
PE	≤ 0.001 (2)
DPPE	0.015 ± 0.006 (3)
20% PS/80% DPPC	0.02 ± 0.006 (3)
33% OP/67% DPPC	≤ 0.001 (2)
10% Glucocerebroside/90% DPPC	0.01 ± 0.007 (2)
25% DPG/75% DPPC	0.058 ± 0.022 (4)
10% Ganglioside/90% DPPC	0.02 ± 0.015 (5)
25% PI/75% DPPC	0.16 ± 0.07 (4)
5% PIP/95% DPPC	0.31 ± 0.023 (3)
40% Soybean PC/40% PE/20% PI	0.82 ± 0.022 (3)
40% Soybean PC/40% PE/20% PS	0.08 ± 0.06 (3)

Values shown are mean conductance ± s.e.m. divided by the mean conductance for asolectin (number of experiments in parentheses). All conductances were obtained by incubating the membrane in the presence of DT for 4 min at 0 mV applied potential. Then, the potential was switched to -30 mV, and the conductance measured 1 min later. Asolectin membranes were generally treated with 100 ng ml⁻¹ of DT; other membranes often needed higher DT concentrations. In these cases, the rate of conductance increase was taken as proportional to the square of the DT concentration^{7,11}. Other conditions were as indicated in Fig. 1 legend. PE, PS, PI, PIP and soybean PC were obtained from Sigma, diphytanoyl phosphatidylcholine (DPPC) from Avanti, glucocerebroside and DPG from Supelco; OP was from Hooker Chemical. Gangliosides (mixed bovine brain) were the gift of E. Yavin; HPLC-purified soybean PC was given by M. Conrad. Lipids were dissolved in CHCl₃ (PIP in water-saturated CHCl₃) or CHCl₃/MeOH (2:1), and stored at -80 °C until use.

The effect of phosphoinositides seems to be due to an interaction of the inositol phosphate moiety with DT which is satisfied even if the inositol is added to the solution in contact with the membrane. Figure 2b shows the effect of adding inositol hexaphosphate (IHP, phytic acid) to the compartment on the toxin-free side of a PS-containing membrane. A current increase was apparent after DT addition only after IHP was added to a concentration of 0.5 mM; further addition of IHP further increased the conductance. In membranes untreated with DT, IHP had no effect on membrane conductance, and addition of IHP to the DT-containing side also had no effect (Fig. 3). The effect of IHP was the same in the presence of 1 mM EGTA or 1 mM Ca²⁺, indicating that this effect is not due to any Ca²⁺ bound to the phosphate groups of the IHP. Rather, the inositol phosphate moiety seems to be essential for channel formation.

It is known that DT in solution binds IHP together with nucleotides and other polyphosphates^{14,15}. Thus, the inositol phosphate effect could conceivably be due to its binding to the DT phosphate binding site, thereby anchoring the DT in a transmembrane conformation. If this were the case, presumably any polyphosphate should have the same effect. However, ATP, when added to the *trans* side of the membrane, does not affect DT channel formation (data not shown). Thus, the effect is specific: only phosphoinositide (or inositol phosphate) on the opposite side of the membrane induces DT channel formation.

We suggest a mechanism in which the DT first binds to the membrane surface, and then extends a process across the

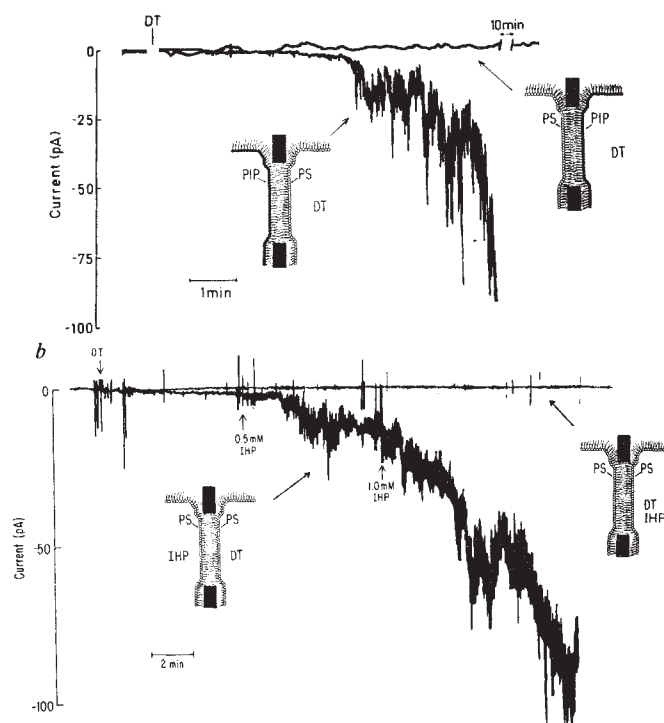


Fig. 2 *a*, Membrane currents through two asymmetric membranes treated with DT. The membranes were formed from two monolayers of different composition: one monolayer contained 10% PS and 90% PC, the other contained 5% PIP and 95% PC. The upper and lower traces show the current through the membrane when DT was added to the solution on the PIP-containing and PS-containing side, respectively. The applied potential was -30 mV throughout. DT concentration, 100 ng ml⁻¹. *b*, Membrane current through two symmetrical membranes in the presence of IHP. The membrane composition was 20% PS, 80% PC. The addition of DT to a concentration of 100 ng ml⁻¹ caused no significant increase in conductance in either membrane. The lower trace shows the current after IHP (Sigma) was added to the side of the membrane opposite to that to which DT was added, and the upper trace shows the current obtained when IHP was added to the same side of the membrane as DT. The voltage was kept constant at -30 mV for both traces.

membrane core which probes the other side. If the DT finds phosphoinositide, and binds to it, anchoring it in place and allowing channel formation to occur. Thus, the critical steps in intoxication may be as follows. DT binds to a cell-surface receptor and is internalized. Once in the endosome, the acidic environment induces a conformational change in DT that, in concert with the membrane potential, promotes the binding and insertion of DT into the endosomal bilayer. The consequent binding to phosphoinositides anchors DT, allowing channel formation and, concomitantly, the translocation of the enzymatic moiety of DT into the cytoplasm. The notion that translocation of proteins across membranes involves conformational changes in the protein and the insertion of portions of the

polypeptide chain has been suggested in a variety of contexts¹⁶⁻¹⁹. The requirements for insertion of the 'signal sequence' of some polypeptides are very similar to the requirements for DT channel formation²⁰⁻²². The remarkable observation that channel formation is facilitated by the presence of inositides in the transmembrane monolayer suggests that specific polypeptide-lipid interactions can play a part in protein translocation.

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Genetic recombination and directional selection for DDT resistance in *Drosophila melanogaster*

Phillip B. Flexon

School of Medicine, Case Western Reserve University, Cleveland, Ohio 44106, USA

Charles F. Rodell*

Department of Biology, College of St Benedict-St John's University, Collegeville, Minnesota 56321, USA

It is generally agreed that recombination offers a long-term evolutionary advantage^{1,2}. It is argued, however, that because genetic variability exists for recombination rates in natural populations, response to immediate demands for adaptation should result in reduced recombination regardless of the long-term consequences. Nevertheless, genetic recombination persists. Numerous models have been developed to examine this fact^{1,2}. There exists, however, a notable lack of empiricism on the recombination question. Here we demonstrate in *Drosophila* that the response of a polygenic character (DDT resistance) to directional selection is significantly correlated with the genetic variability generated by recombination to bring about a corresponding increase in recombination rate. We draw attention to the relationship of these results to existing models, notably the 'hitch-hiking' model³.

Twenty-eight single female lines of *Drosophila melanogaster*, free from chromosomal inversions, were cultured from individuals collected in central Tennessee. On 8 August 1978, five inseminated females from each line were combined to establish two control populations (C1 and C2) and three selected populations (R1, R2 and R3). Populations were maintained in population units consisting of two 1/2-pint bottles united by a 7.6-cm piece of hose⁴. The cultures were maintained at 25 ± 1 °C and the bottles replaced alternatively every 2 weeks throughout the study. On 20 August 1978, selection for DDT (1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane) resistance was initiated on populations R1, R2 and R3 by placing a 2.5 × 7.5 cm

strip of filter paper impregnated with DDT on the medium surface in each fresh bottle. Selection was carried out by gradually increasing the amount of DDT from 0.1 mg to 190.0 mg over the next 555 days.

Three kinds of data were gathered from these populations. Population levels of DDT resistance were measured to demonstrate that directional selection had occurred; cross-over frequencies for portions of chromosomes I, II and III were determined to detect differences in recombination rates between initial, control and selected populations; and individual chromosomes from the selected populations were tested for resistance. The level of chromosomal resistance attained was then related to changes in chromosomal recombination rates.

DDT resistance was measured by subjecting the daughters of sampled females to a knockdown test⁴. After 330 days of selection, populations R1, R2 and R3 achieved resistance levels 10-40 times greater than those of the control populations, demonstrating that selection was effective. The median effective dose (ED₅₀) for DDT was 5.3 and 6.4 mg for populations C1 and C2, respectively, and 207.5, 160.4 and 54.1 mg for R1, R2 and R3, respectively⁵. Population levels of DDT resistance were not tested again even though selection continued for another 165 days.

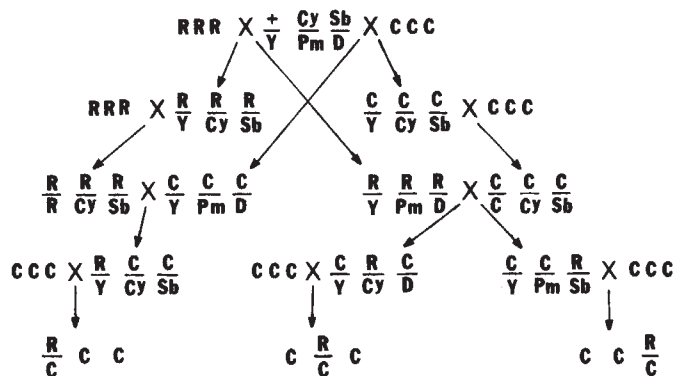


Fig. 1 Mating scheme that allows for the isolation of females possessing individual I, II or III chromosomes from the selected population. R, chromosome from the selected population; C, chromosome from the control population; +, wild-type I chromosome; Sb, curly; Y, Y chromosome; Pm, plum; and D, dichaete.

* To whom correspondence should be addressed.

Table 1 Recombination rates for portions of chromosomes I, II and III in control and selected populations of *Drosophila melanogaster*

Population	Chromosome	Mean recombination index $\pm 95\%$ confidence interval	No. of females sampled from each population	No. of individuals counted	Months of DDT selection before recombination tests
Wild type	I†	0.286 $\pm$ 0.041	10	1,500	0
C1		0.277 $\pm$ 0.027	25	991	19*
C2		0.290 $\pm$ 0.026	25	962	18*
R1		0.276 $\pm$ 0.020	25	986	19
R2		0.283 $\pm$ 0.021	24	958	19
R3		0.288 $\pm$ 0.026	24	1,119	18
C1	II‡	0.330 $\pm$ 0.020	36	1,910	12*
C2		0.309 $\pm$ 0.035	26	1,313	12*
R1		0.369 $\pm$ 0.023	23	1,675	11
R2		0.373 $\pm$ 0.031	20	1,063	12
R3		0.398 $\pm$ 0.021	37	1,899	12
Wild type	III§	0.056 $\pm$ 0.013	12	1,153	0
C1		0.059 $\pm$ 0.014	20	976	13*
C2		0.057 $\pm$ 0.014	30	1,575	13*
R1		0.096 $\pm$ 0.020	33	1,914	13
R2		0.098 $\pm$ 0.022	24	1,323	14
R3		0.094 $\pm$ 0.018	25	1,359	14

The 'recombination index' represents the number of recombination events observed divided by the total number of possible recombination events.

* Populations C1 and C2 were not exposed to DDT but in all other aspects were treated identically for the period of time shown.

† Recombination index determined for markers *w, m, f*.

‡ Recombination index determined for markers *dp, cn, bw*.

§ Recombination index determined for markers *wmf*.

§ Recombination index determined for markers *G1, Sb*.

The recombination data are summarized in Table 1. Following selection, recombination rates between loosely linked markers were determined for the major chromosomes in all populations. Initial rates were also determined for chromosomes I and III. It became necessary to change the markers for chromosome II after the experiment began; because of this change there are no initial data for that chromosome. These rates were obtained by crossing daughters of sampled females to marker stock males. F_1 daughters were again crossed to marker males and F_2 individuals scored for recombinant phenotypes. The data are expressed as a recombination index, representing the number of recombination events observed divided by the total number of possible recombination events. For chromosomes I and II with three markers per chromosome, the recombination index is computed by dividing the number of recombination events among F_2 individuals by twice the total number of F_2 individuals, as it is possible to observe a maximum of two recombination events in each individual. Chromosome III data are based on two markers; thus, the recombination index is the number of recombination events observed divided by the total number of F_2 individuals. One-way analysis of variance of the recombination index values for each chromosome indicated significant differences between populations for chromosomes II ($F_{4,137} = 8.64$; $P < 0.001$) and III ($F_{5,138} = 3.34$; $P < 0.01$) but no significant differences for chromosome I ($F_{5,127} = 0.45$; $P > 0.10$). Least-squares difference tests for the chromosome II values indicated no significant difference between the mean recombination index values for populations C1 and C2 and no significant differences between the means of the selected populations. However, each of the selected populations has a recombination rate ratio index significantly greater than each control population ($P < 0.05$ for all comparisons). The same results were obtained for the chromosome III data, that is $P < 0.05$ for each R population compared with each C population. These results indicate that a change in the level of DDT resistance brought about by directional selection was accompanied by a significant increase in the intrachromosomal recombination rate for portions of two of the three major chromosomes in this species.

The final step consisted of testing the level of DDT resistance for each chromosome separately. The rationale behind this step is as follows. If genes affecting recombination rates are maintained to any significant extent in populations as a result of producing new favourable recombinants, then the recombination genes must be sufficiently linked to these recombinants in order to 'hitch-hike' their way to a higher frequency. As significant increases in recombination frequency were found on chromosomes II and III, the hitch-hiking hypothesis predicts that these same chromosomes would possess the major genetic

component for DDT resistance.

Individuals with the desired combination of unselected (C) and selected (R) chromosomes were obtained from a series of crosses by using inversion-containing marker chromosomes (Fig. 1). Again, females alone were evaluated for resistance by knockdown tests⁴. The results are striking when the chromosomal level of resistance, as indicated by the ED_{50} values⁵, is compared with the ratio of selected chromosome recombination rate to that of the control chromosomes (Fig. 2), that is, chromosomal response for DDT resistance compared with chromosomal response in recombination rate. The greater the chromosomal response in recombination rate, the greater was the chromosomal response for DDT resistance; this significant positive correlation supports the hitch-hiking hypothesis.

It is necessary to consider whether the increases in recombination rate observed in the selected populations might be due to some factor other than selection. It seems unlikely that mutation could have caused this change. Studies on the

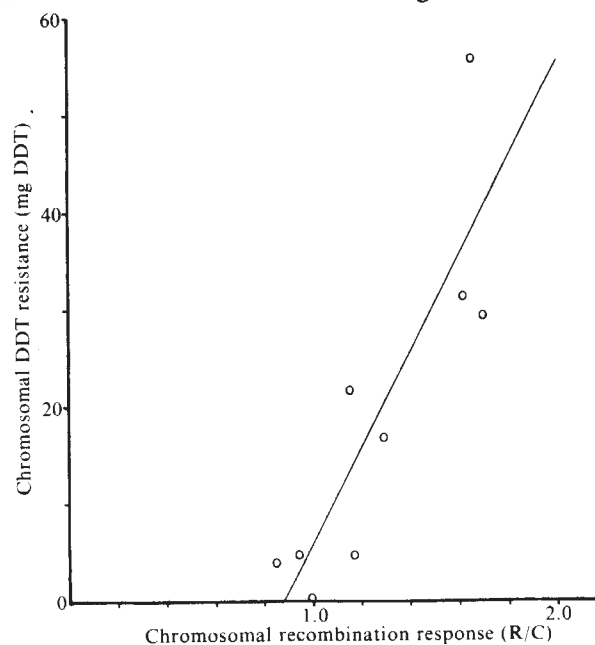


Fig. 2 The relationship between the level of chromosomal DDT resistance and chromosomal recombination response. The latter is taken as the ratio of the R population recombination index to the mean recombination index of the two C populations for a given chromosome: $y = 49.7x - 43.7$; $r_{yx} = 0.86$, $P < 0.01$ for H_0 : $r_{yx} = 0$.

mutagenic ability of DDT indicate that it is either non-mutagenic⁶⁻⁹ or is only a weak mutagen^{10,11}. It is possible that the increases were brought about by genetic drift. Because the selected populations were smaller in number than the control populations, the high rate alleles may have drifted to high frequencies. However, the fact that all populations responded in the same way favours directional selection as the major determinant of recombination rate increases.

These results are of interest in two respects: they provide the first demonstration of increased recombination correlated with the response to directional selection by a different quantitative character, and confirm the proposal that recombinant genes generating genotypes favoured by selection can 'hitch-hike' to increased allele frequencies³. For hitch-hiking to be a major factor in maintaining recombination in natural populations, these populations must experience either continuing directional selection or normalizing selection with a moving optimum¹². Whenever a balancing selection model is analysed, selection for decreased recombination results^{12,13}. The question becomes one of the relative importance of normalizing selection with a stable optimum as opposed to selection where the optimum is changing with time. The stabilizing selective mode might be favoured by those who prefer to think of adaptation as a response primarily to abiotic stresses such as temperature and humidity, or intraspecific interactions such as courtship, which present a more or less fixed range in which the species must adapt. It can be argued that adaptations to interspecific biotic stresses may be more important and more prevalent than those to abiotic stress¹⁴⁻¹⁶. Such co-evolutionary relationships are more likely to involve selection of the kind favouring recombination.

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Maintenance of broad host range plasmid RK2 replicons in *Pseudomonas aeruginosa*

Christopher M. Thomas, Atta A. K. Hussain & Christopher A. Smith

Department of Genetics, University of Birmingham, PO Box 363, Birmingham B15 2TT, UK

Certain members of the *Escherichia coli* plasmid incompatibility group P are capable of efficient transfer between and maintenance in most Gram-negative bacterial species¹⁻⁴. Of fundamental interest is whether this broad host range results from differences between the mechanisms of replication and partitioning during cell division used by IncP plasmids and by narrow host range plasmids. The genetic regions of IncP plasmid

RK2 (similar or identical to RP4 and RP1) required for plasmid maintenance in *E. coli* have previously been defined as a vegetative replication origin (*ori_{RK2}*) and a gene specifying a positively required *trans*-acting product (*trfA*)⁵⁻⁸. Here we have tested various mini RK2 replicons for their ability to become established after transformation into *Pseudomonas aeruginosa* PAO as a model non-*E. coli* host. We chose *P. aeruginosa* for this purpose as it is a Gram-negative species relatively phylogenetically distant from *E. coli*. Our studies have defined a plasmid segment that contains a region which must be linked in *cis* to the *ori_{RK2}* replicon to allow mini RK2 plasmid maintenance in *P. aeruginosa* PAO and suggest that RK2 may carry genes which adapt the basic replicon to allow autonomous maintenance in specific groups of hosts.

A simplified physical and genetic map of plasmid RK2 is shown in Fig. 1. A segment of the plasmid genome (from coordinates 54.0 to 56.0 kilobases (kb)) has been designated the *trfB* region because experiments carried out in *E. coli* indicate that it contains a gene, *trfB*, that specifies a *trans*-acting product acting in plasmid replication or maintenance when the *trfB* region is part of the RK2 replicon^{7,8}. The requirement for at least part of this region for establishment in *P. aeruginosa* has been suggested by the inability of unstable RP4 mutants with transposon Tn7 inserted in the *trfB* region to transfer from *E. coli* to *P. aeruginosa*⁹. Also, a mutation has been described¹⁰ that renders RP4 temperature-sensitive for maintenance in *P. aeruginosa* but not in *E. coli* and which physically maps in a plasmid segment that contains *trfB* but not *trfA* or *ori_{RK2}*.

We have analysed the *trfB* region requirement by testing the ability of mini replicons derived from RK2 by *in vitro* recombination and isolated from *E. coli* to be introduced by transformation into the restriction-deficient *P. aeruginosa* PAO1161. The structures of the plasmids we have used are shown in Fig. 2 and the results of transformation in Table 1. If a plasmid seemed to become established in PAO1161, plasmid DNA was isolated by the alkaline extraction method of Birnboim and Doly¹¹ and its structure verified by restriction endonuclease digestion pattern analysis. Our initial observation was that whereas mini RK2 replicons carrying all three regions, *ori_{RK2}*, *trfA* and *trfB* (pRK248), were capable of establishment in PAO1161,

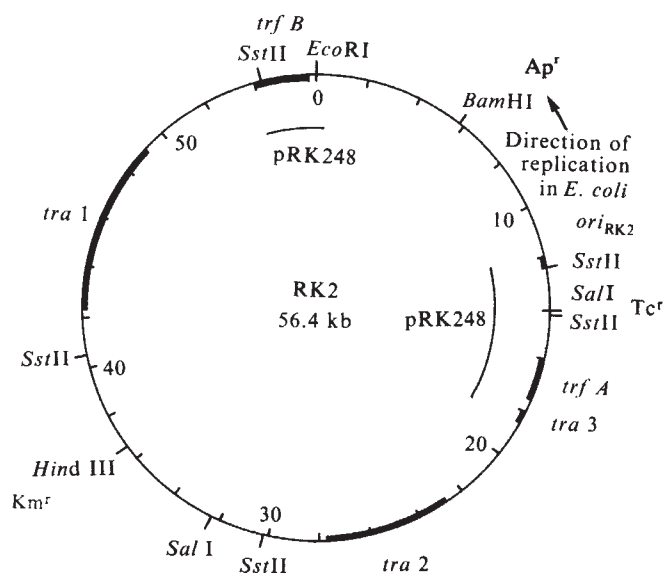


Fig. 1 Simplified physical and genetic map of broad host range plasmid RK2 (RP4 and RP1). Only a few restriction sites are shown as reference points. The single *EcoRI* site is used as the origin of kilobase pair (kb) coordinates. The regions specifying resistance to ampicillin (*Ap^r*), tetracycline (*Tc^r*) and kanamycin (*Km^r*) are shown together with regions (indicated by heavy lines) specifying genes required for conjugal transfer (*tra*), the vegetative replication origin (*ori_{RK2}*) and regions containing genes specifying *trans*-acting replication or maintenance functions (*trf*). The segments of RK2 present in the mini replicon pRK248 are shown.

Table 1 Results of transforming *P. aeruginosa* PAO1161 (Leu⁻Res⁻Mod⁺) with DNA of mini RK2 replicons isolated from *E. coli* K12 C600 Δ trpE5

Plasmid	Average no. of transformants per plate*		
	PAO1161	PAO1161 (pCT407)	PAO1161 (pCT408)
pRK248	2,000	89	2,000
pCT201	0	0	0 (6)†
pCT398	1,500	104	1,500
pCT427	200	14	1,000
pCT428	600	20	1,200

PAO1161 was made competent for transformation by pelleting 10 ml of late exponential phase culture ($A_{1cm,625} \approx 1.0$) grown in L broth (10 g tryptone, 5 g yeast extract, 5 g l⁻¹ NaCl) at 30 °C, washing with 4 ml ice-cold 100 mM MgSO₄, incubating in 4 ml ice-cold 100 mM CaCl₂ for 20 min and finally resuspending in 1 ml ice-cold 100 mM CaCl₂. Approximately 1 µg of DNA was mixed with 100 µl of competent bacteria and incubated at 0 °C for 60 min. After 2 min at 42 °C, 1.0 ml of L broth was added and after 120 min at 30 °C, 9 ml of L broth with tetracycline to 10 µg ml⁻¹ was added. After overnight incubation at 30 °C, 0.2-ml samples were plated on L agar (L broth with 15 g l⁻¹ agar) with 100 µg ml⁻¹ tetracycline and incubated at 30 °C. pCT407 is an IncQ plasmid, R300B⁹ (conferring resistance to streptomycin and sulphonamide) linked to *trfB*. pCT408 is R300B linked to *trfA* and *trfB*.

* If Tc^r transformants were obtained the presence of independent plasmid DNA was verified by extraction and examination of plasmid DNA¹¹.

† On one occasion Tc^r transformants were obtained but examination of plasmid DNA indicated that the tetracycline resistance was conferred by a recombinant between pCT201 and pCT408.

pCT201, which lacks all but 150 base pairs of the *trfB* region, was not. The *trfB* region was reinserted into pCT201 by *in vitro* recombination and the resultant plasmid (pCT398) was found to be capable of transforming and being maintained in PAO1161 (pCT398 is lost from PAO1161 at ~4–5% per generation in the absence of selection for plasmid markers). Therefore, the inability of pCT201 to transform PAO1161 is

due to the lack of the *trfB* region rather than to a secondary defect located elsewhere on the plasmid.

To determine whether the requirement for all or part of the *trfB* region is solely to provide a *trans*-acting product necessary for *ori*_{RK2} or *trfA* function in PAO1161, we cloned both *trfB* alone (pCT407) and *trfA* together with *trfB* (pCT408) into IncQ broad host range plasmid R300B by *in vitro* recombination and introduced these plasmids into PAO1161. The presence of *trfA* and *trfB* in the PAO1161 host did not significantly increase its ability to be transformed by pCT201. In one experiment, transformants were obtained but the restriction pattern of the plasmid DNA isolated from these transformants suggested that they contained a hybrid plasmid resulting from recombination between pCT201 and pCT408 after entry rather than pCT201 and pCT408 existing as separate plasmids. These results were interpreted to mean that part or all of the *trfB* region is needed linked in *cis* to pCT201 to allow establishment and maintenance in PAO1161 rather than simply for DNA entry into competent bacteria. Insertion of only part of the *trfB* region into pCT201 also restores the plasmid's ability to transform PAO1161, although the stability of these plasmids (pCT427 and pCT428) without selection for plasmid markers in PAO1161 is less than for pCT398 (pCT427 is lost at 5–10% per generation and pCT428 at 8–12% per generation). The limits of the essential segment of the *trfB* region are not known. The orientation of the inserted *trfB* segment relative to *ori*_{RK2} and *trfA* (pCT427 and pCT428) does not matter so it seems unlikely that the *trfB* region is providing a transcription promoter active in *P. aeruginosa* that is necessary for *ori*_{RK2} or *trfA* function.

As these results indicate that *ori*_{RK2} and *trfA* as defined in *E. coli* are not sufficient for establishment and maintenance in *P. aeruginosa*, it could be that one or both of these regions is not needed in *P. aeruginosa*. However, we were unable to transform a plasmid containing *trfA* and *trfB* but lacking *ori*_{RK2} (pCT141) into PAO1161. Also RP4.Cole1 hybrids with transposon Tn7 inserted either into *ori*_{RK2} or *trfA* (obtained from A. Stepanov) are incapable of conjugal transfer from *E. coli* to *P. aeruginosa* and *trfA* temperature-sensitive mutants are unstable at the non-permissive temperature in *P. aeruginosa*.

Fig. 2 Structure of mini RK2 replicons used to investigate the requirement for segments of the *trfB* region in *P. aeruginosa*. Sites for restriction endonuclease cleavage are labelled as follows: *Bam*HI, B; *Eco*RI, E; *Hind*III, H; *Sal*I, Sa; *Sst*II, Ss; *Xho*I, X. The segments of DNA derived from RK2 are shown as solid lines, non-RK2-derived DNA as open lines. The circular plasmids are represented as linear molecules with the break point defined by the *Eco*RI site for pRK248 and the other plasmids aligned with respect to the RK2 maintenance functions. pRK248 (9.6 kb) was produced indirectly from RK2 by partial digestion with restriction endonuclease *Hae*II and ligation⁷ (Fig. 1). pCT201 (10.5 kb) was constructed by ligating two *Hind*III fragments, one containing Tc^r, *trfA* and ~150 base pairs of the *trfB* region and the other Ap^r and *ori*_{RK2}. pCT398 (12.0 kb) was constructed by linking pCT201 to a hybrid plasmid carrying *trfB* and then carrying out *Sst*II partial digestion to delete the intervening DNA segment and join the partial *trfB* segment already present in pCT201 to the rest of the region containing *trfB*. pCT427 and pCT428 were constructed by replacing the Ap^r *Bam*HI to *Eco*RI segment of pCT201 by a Km^r *Bam*HI to *Eco*RI segment from hybrid plasmids containing segments of a mini RK2–Km^r plasmid inserted into the *Cla*I site of pBR322. The two derivatives used have Km^r and part of the *trfB* segment as shown inserted in opposite orientations relative to the *Bam*HI and *Eco*RI sites. pCT141 (11.8 kb) is a hybrid with a Tc^r *Hind*III fragment carrying *trfA* and *trfB* inserted into a chloramphenicol resistance-conferring (Cm^r)Tc^s derivative, pDS3, of pACYC184.

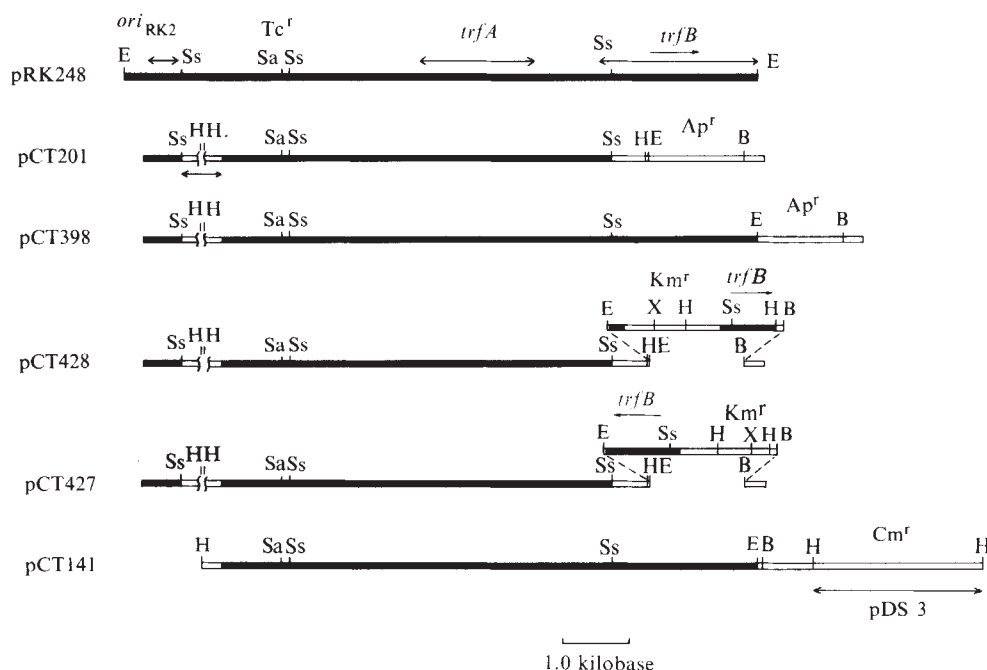
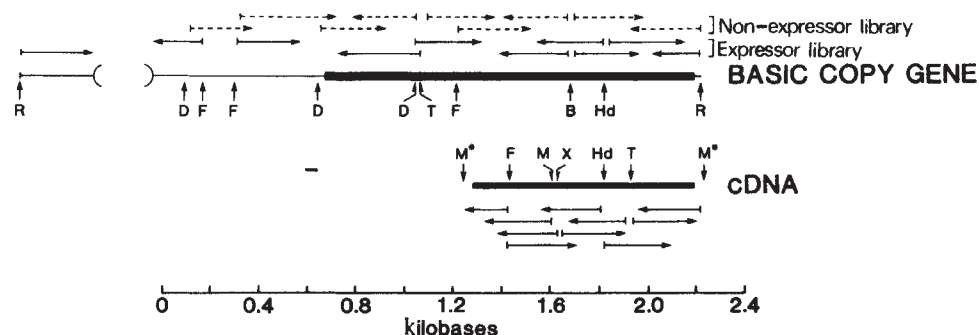


Fig. 1 The regions sequenced (indicated by horizontal arrows) in the BC gene and the cDNA for the VSG of ILTAT 1.1. The BC gene (thick line of upper diagram) is on a 2.8 kb *EcoRI* fragment that was initially identified in bacteriophage λ Charon 4A libraries of ILTAT 1.1 (expressor library) and ILTAT 1.3 (non-expressor library) genomic DNA. The 2.8 kb *EcoRI* fragment isolated from a phage clone of each library was subcloned into the *EcoRI* site of pBR322 for sequence determination using the Maxam-Gilbert technique¹⁸. The dashed arrows show the locations of sequences determined in the 2.8 kb fragment isolated from the ILTAT 1.3 (non-expressor) library and the solid arrows sequences determined in the same fragment isolated from the ILTAT 1.1 (expressor) library. Parentheses show a region of about 250 bp where the sequence was not obtained. The bottom diagram shows the restriction sites used to determine the sequence of the cDNA. In both diagrams, arrows pointing to the left and right are sequences obtained on the upper and lower strands respectively. Restriction sites labelled by the DNA polymerase I repair reaction³³ for Maxam and Gilbert sequencing were *EcoRI* (R), *DdeI* (D), *HinfI* (F), *TaqI* (T), *BamHI* (B), *HindIII* (Hd), *XbaI* (X), *MspI* (M) and *MspI* sites in pBR322 (M*).



library of ILTAT 1.1 DNA cloned in bacteriophage λ Charon 28 have not been successful (J. R. Young, P. A. O. Majiwa, P. T. Englund, R. O. Williams and J.E.D., unpublished) for unknown reasons that may be related to flanking repetitive sequences and/or the chromosomal location of the ELC¹⁷. However, the 2.8 kb *EcoRI* fragment containing the BC gene was found in bacteriophage libraries of all four ILTAT DNAs. This fragment was isolated from a phage clone of the ILTAT 1.1 DNA (expressor) library and an ILTAT 1.3 DNA (non-expressor) library and both fragments were subcloned into the *EcoRI* site of plasmid pBR322 for sequence determination via the Maxam and Gilbert technique¹⁸. Figure 1 shows the strategies used to determine these two genomic DNA sequences and the sequence of an ILTAT 1.1 VSG cDNA. More than 95% of the BC gene sequence was determined in the 2.8 kb fragment from the non-expressor library (dashed arrows) and about 90% in the fragment from the expressor library (solid arrows), so that taken together the complete sequence of the BC gene could be deduced. No differences were detected in the 2,100 nucleotides of each of the two genomic fragments that were determined separately, but many differences with the ILTAT 1.1 VSG cDNA were found.

Figure 2 compares the BC gene and cDNA sequences. Boxes indicate the non-homologous nucleotides. Also shown is the VSG amino acid sequence predicted by the BC gene sequence and those amino acids affected by the cDNA differences. The 696 nucleotides preceding the BC gene contain many termination codons in all three reading frames. A potential initiator methionine codon at position 697 is followed by an open translation reading frame of 471 codons which would code for a protein in the size range reported for different VSGs¹²; this suggests that the gene is not split by an intervening sequence. The first 15 codons specify a rather hydrophobic N-terminus which may be a signal peptide, although this cannot be confirmed on the basis of nucleotide sequence alone. The comparison with the cDNA sequence starts at nucleotide position 1,287 and in the next 450 nucleotides, there are 18 nucleotide differences (4% of the nucleotides) which affect 13 of the amino acids specified (9% of the amino acids). There is no obvious pattern to these changes with respect to either relative position or purine versus pyrimidine replacements. No differences occur in the next 324 nucleotides but then 11 changes occur in the last 46 nucleotides before the termination codon. The two sequences are very different in the 3'-non-translated region.

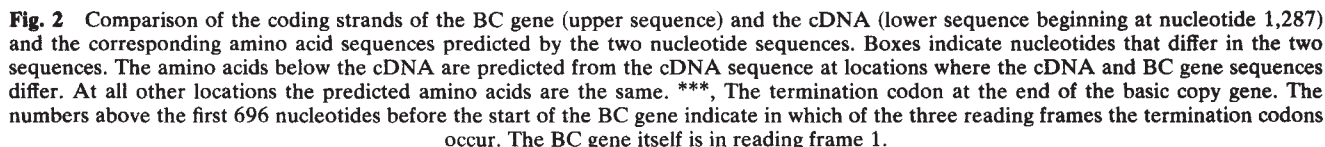
To ensure that these nucleotide differences were not the result of copying mistakes by enzymes used *in vitro* to construct the cDNA, portions of the sequences of two additional cDNAs containing the ILTAT 1.1 VSG coding sequence were determined (not shown). The same point changes were also present in the other two cDNAs, reducing substantially the possibility that they were the result of copying or cloning mistakes. Inter-

estingly, one of the cDNAs did contain only four adenosines at positions 1,689–1,693 (and four thymidines in the complementary strand) in comparison with the five adenosines at this location in the other two cDNAs and the two genomic fragments. Since other cloned cDNAs with sequence errors have been reported previously^{19,20}, it seems likely that this single base pair deletion is the result of an enzymatic mistake during the synthesis of that cloned cDNA molecule *in vitro* or during its replication after transformation into *Escherichia coli*. Another less likely possibility is the occurrence of incorrect VSG mRNA transcription *in vivo*. At the very least, this observation demonstrates the hazard of relying on a single cloned cDNA molecule to determine a coding sequence.

Further confirmation that the point changes occur during the generation of the ELC *in vivo* was derived from previous Southern hybridization data which predicted that the ILTAT 1.1 BC gene contains a *BamHI* site while the ELC gene does not⁹. And indeed, the sequence of the BC gene has a *BamHI* site at positions 1,697–1,702 while the cDNA sequence does not because of a single nucleotide difference at position 1,700. Similarly, Southern hybridizations demonstrate that if there are two copies of the BC gene because the parasite is diploid²¹, then both copies contain a *BamHI* site while the ELC does not. Thus it appears that these nucleotide changes occur during the duplication and translocation events that generate the ELC from the pre-existing BC.

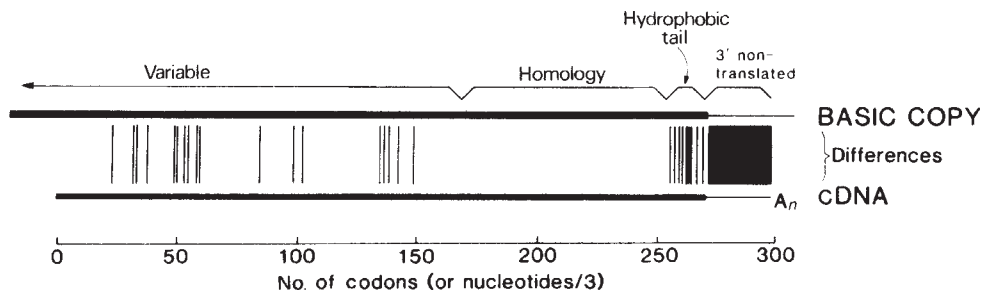
Figure 3 shows the locations of the nucleotide changes with respect to the different VSG regions specified. Eighteen changes occur in the variable region, none in the homology region, eleven in the hydrophobic tail and many in the 3'-non-translated region which possesses no more homology than would be expected of two random 80% (dA + T) sequences (49 differences in 84 nucleotides = 58%).

An alternative explanation of the results is the loss of a second BC gene concomitant with the appearance of the ELC but this seems unlikely for at least two reasons. Unfortunately this possibility cannot be formally eliminated, however, because ILTAT 1.1 is the first clone in the ILTAT series to be maintained (R. O. Williams, personal communication) so that genomic DNA from a trypanosome clone preceding ILTAT 1.1 is unavailable. However, when ILTAT 1.1 cDNA is used as a radioactive probe in Southern hybridizations with genomic DNA from *T. brucei* clones unrelated to the ILTAT series, only one BC gene is detected (not shown). In addition there is no evidence that the appearance of ELCs of other VSG genes being studied by other research groups is accompanied by the loss of a BC gene^{5-8,14}. Thus if a second BC gene existed, the simultaneous loss of one BC gene and appearance of an ELC would represent a third type of DNA rearrangement associated with VSG expression. Furthermore, a hypothetical second BC gene still would not account for the confinement of random



Although the mechanism(s) that generate(s) these mutations may be obscure, their biological significance is not. In influenza virus, a difference in less than 5% of the amino acids of the surface antigen is sufficient to change the antigenic specificity²². Therefore the trypanosome mutations could potentially increase the repertoire of immunologically distinct VSGs that are coded for by a limited number of different genes. They may in fact generate virtually an infinite number of potential VSGs for the trypanosome in the same way that somatic mutations are responsible, in part, for the immense diversity of antibodies generated by lymphocytes in the immune response (for a recent review, see ref. 10). These somatic mutations in the V segments of antibody genes occur in both the hypervariable regions and the framework residues and sometimes occur during the isotype switch from one constant region gene to another²³, so they may be introduced by a different and less localized mutation than the changes described here in the generation of the ELC. The

Fig. 3 Schematic comparison of the sequences of the BC gene and cDNA showing the 3'-non-translated region and poly(A)_n, the locations of coding portions that specify the various regions in the VSG (variable, homology and C-terminal hydrophobic tail regions) and the locations of nucleotide differences between the basic copy gene and the cDNA (vertical lines and solid rectangle).



two mutational processes are similar, however, in that there is no simple preference for purines or pyrimidines and some mutations are silent. Both processes are clearly important for generating the diversity of amino acid sequences found in the two types of protein.

Van der Ploeg *et al.*¹⁷ have recently reported from Southern hybridization data (see below) that the DNA segment duplicated and translocated to generate the ELC of another VSG gene, VSG 117, begins 1–2 kb before the start of the BC gene. If the ILTAT 1.1 system is similar, then the 696 nucleotides reported here that precede the initiator methionine codon probably do not contain the 5'-recombination site. Indeed, by inspection or computer searches, we have been unable to identify a sequence that resembles switch sites of immunoglobulin genes or the yeast mating-type locus^{24–27}, sequences common to other eukaryotic transposable elements^{28,29} or bacteriophage 'Chi' site sequences³⁰. The sequence of another 300 nucleotides upstream has been determined (from the left-hand *EcoRI* site shown in Fig. 1) but is not shown in Fig. 2 because it is not contiguous with the sequence presented. This 300 nucleotide sequence, which is within 1–2 kb in front of the BC gene, also does not contain an obvious potential recombination site.

Bernards *et al.*³¹ have reported a partial sequence comparison of the BC gene of VSG 117 and its corresponding cDNA which includes the hydrophobic tail and 3'-non-translated regions, but not the homology region or variable region. The VSG 117 system, like ILTAT 1.1, exhibits nucleotide changes in the hydrophobic tail region. Some of these changes, however, are nucleotide additions in the cDNA relative to the BC gene, a feature not present in the ILTAT 1.1 system. Another difference is that the VSG 117 system exhibits substantially more homology in the 3'-non-translated regions of the cDNA and BC gene than does the ILTAT 1.1 system. The significance, if any, of these differences is not known. Recently, Frasch *et al.*³² reported Southern hybridization studies that suggested the occurrence of 'local hypermutagenesis' in subsets of closely related (cross-hybridizing) VSG genes which may correspond to the localized mutagenesis observed in the variable region of the ILTAT 1.1 BC gene (Fig. 3). Thus the occurrence of mutations during the generation of ELCs may be part of a general property of the entire VSG gene family, further demonstrating the intricate mechanisms that evolved in trypanosomes, permitting them to evade their hosts' immune defences.

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Amplification of endogenous *myc*-related DNA sequences in a human myeloid leukaemia cell line

Steven Collins

Veterans Administration Hospital, 4435 Beacon Avenue South, Seattle, Washington 98108, USA

Mark Groudine

Fred Hutchinson Cancer Research Center, 1124 Columbia Street, Seattle, Washington 98104; Department of Radiation Oncology, University of Washington Hospital, Seattle, Washington 98195, USA

Malignant transformation of cells by acute transforming RNA tumour viruses is mediated by the expression of certain specific proviral DNA sequences ('oncogenes'). These sequences have been well characterized and, in many cases, molecularly cloned^{1–8}. These viral oncogenes are related to similar genes found in normal uninfected cells^{9,10}. Moreover, these particular sequences are highly conserved in evolution^{4,11}, suggesting that these genes have an important, albeit unknown, role in normal cell function. It has been suggested that an increased dosage of products of such endogenous oncogenes may be responsible for malignant transformation^{10,12,13}. For example, increased expression of the endogenous chick *c-myc* oncogene has been observed in avian leukosis virus-induced transformation of chick bursal lymphocytes¹². Here we demonstrate that sequences in normal human DNA homologous to the avian *myc* oncogene are present in multiple copies in the chromosomal DNA of the human leukaemia cell line HL-60. Other transformation-specific genes derived from the Abelson leukaemia virus⁴ and feline sarcoma virus⁶ are not amplified in HL-60. This *myc*-related gene amplification is not present in other cultured human myeloid leukaemia cells, including K-562¹⁴ and KG-1¹⁵.

The HL-60 cell line is a culture of malignant promyelocytes derived from the peripheral blood leukocytes of a patient with

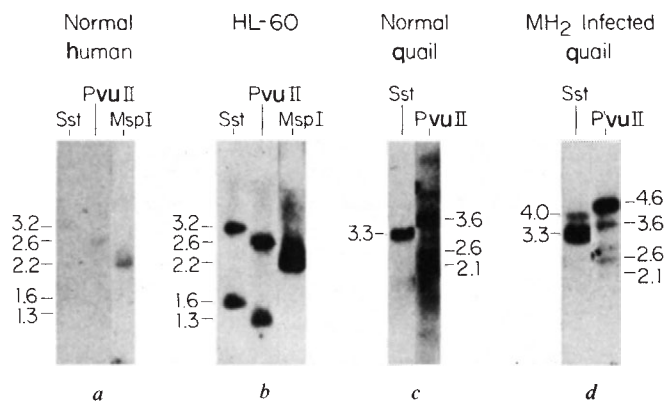


Fig. 1 *myc*-related sequences in DNA of HL-60, normal human and avian cells. Cells analysed included: *a*, normal human cells obtained by PHA stimulation of peripheral blood mononuclear cells from a normal human donor; *b*, HL-60 cells, passage 40, cultured as previously described¹⁶; *c*, normal quail embryo fibroblasts; *d* a cloned subline of quail embryo fibroblasts non-productively transformed with the MH₂ virus (a member of the MC-29 family of avian retroviruses) and obtained from M. Linial. DNA from these cells were restricted with the designated enzymes, subjected to agarose gel electrophoresis (1.2%) (10 µg of DNA per lane) followed by Southern blot hybridization²⁶ using a ³²P-labelled probe prepared by nick translation²⁷ of the *c-myc* probe. *c-myc* is a 3.3-kb *Sst* fragment obtained from a bacteriophage clone of *c-myc* that was isolated from a library of random chicken DNA fragments. This fragment encompasses almost the entire *c-myc* locus as detailed elsewhere²⁸. The *c-myc* *Sst* fragment was subcloned in a derivative of pBr322 containing a single *Sst* site. Hybridizations were carried out in 50% formamide, 10% dextran sulphate 3 × SSC at 42 °C. Following hybridization, the filters were washed in 0.1% SDS, 0.1 × SSC at 50 °C and then exposed to Kodak XAR-5 film with intensifying screens. All blots were exposed for 72 h. Molecular weights of fragments are given in kilobases.

acute promyelocytic leukaemia¹⁶. DNA samples extracted from cells of this line and from phytohaemagglutinin (PHA) stimulated peripheral blood lymphocytes from a normal donor were digested with restriction endonucleases, separated by electrophoresis on agarose gels and transferred to nitrocellulose. The resultant blots were hybridized to a ³²P-labelled DNA probe specific for the chick *c-myc* gene, the cellular homologue of the transforming sequences of acute transforming retroviruses within the MC-29 family^{17,18}. This *c-myc* probe gave faint but distinct bands when hybridized to various endonuclease digests of normal human DNA (Fig. 1*a*), indicating homology between certain sequences within the human genome and the chick *c-myc* probe, as reported by others¹⁹. From the same blot, bands of identical size were noted in endonuclease digests of HL-60 DNA, but the intensity of hybridization was much greater (Fig. 1*b*). The total amount of normal human and HL-60 DNA loaded in each lane was the same for all DNA preparations (10 µg). Moreover, when the same normal and HL-60 samples were hybridized with other ³²P-labelled probes, bands of similar intensity were noted in both normal and HL-60 DNA (Fig. 2). This indicates that the enhanced hybridization of the *c-myc* probe to HL-60 DNA is not a result of differences in the amount of DNA loaded on the gels.

The intensity of hybridization of the *c-myc* probe to HL-60 DNA is comparable with that noted in DNA extracted from normal avian cells and from avian cells infected with the acute transforming MH₂ virus (Fig. 1*b-d*). However, the similarity in restriction patterns of HL-60 and normal human DNA using the *c-myc* probe (Fig. 1*a, b*) makes it unlikely that the intense hybridization seen with HL-60 DNA is a result of inadvertent infection of HL-60 cells with a retrovirus such as MH₂ containing *myc* sequences or of contamination of HL-60 DNA with avian DNA.

The increased hybridization of the *c-myc* probe to HL-60 DNA compared with normal human DNA could be the result of either multiple copies (amplification) in HL-60 cells of the endogenous human sequences homologous to *c-myc* or to increased homology of these endogenous HL-60 *c-myc*-related sequences to the chick *c-myc* probe. The similarity in restriction enzyme patterns of HL-60 and normal human DNA hybridized to *c-myc* (Fig. 1) indicates that HL-60 and normal human *myc*-related DNA sequences are similar. Therefore, this increased HL-60 hybridization to *c-myc* most probably results from amplification in HL-60 DNA of *c-myc*-related sequences. To confirm this, we 'dot-blotted' serial dilutions of HL-60, normal human and avian DNA onto nitrocellulose as previously described²⁰, hybridized the filter with ³²P-labelled *c-myc* probe, and then estimated the melting point of the resultant hybrids by washing the filters at progressively higher temperatures (Fig. 3). If the enhanced hybridization of HL-60 DNA to the *c-myc* probe results from increased homology of *myc*-related HL-60 sequences to this probe, the melting points of avian and HL-60 *c-myc* hybrids should be similar. Alternatively, if endogenous *myc*-related genes are amplified in HL-60, then the melting points of HL-60 and normal human DNA-*myc* hybrids should be similar and should be lower than that seen with avian DNA. As demonstrated in Fig. 3, the intensity of hybridization of the *myc* probe to equal quantities of HL-60 and avian DNA is similar, but both HL-60 and normal human DNA *c-myc* hybrids are denatured at lower temperatures than avian DNA *c-myc* hybrids. The relative denaturation of both HL-60 and normal human DNA hybrids at a given temperature appears similar (Fig. 3). These results indicate that the enhanced hybridization of the *c-myc* probe to HL-60 DNA is a result of HL-60 amplification of the endogenous human *myc*-related sequences rather than to increased homology of these sequences to the *c-myc* probe. Moreover, one can estimate by comparing the intensity of hybridization of the *c-myc* probe to serial dilutions of HL-60 and normal human DNA (Fig. 3) that *c-myc* related sequences are amplified some 8–16-fold in HL-60 DNA compared with normal human DNA.

Identical restriction fragments are noted when the *c-myc* probe is hybridized to normal human and HL-60 DNA digested

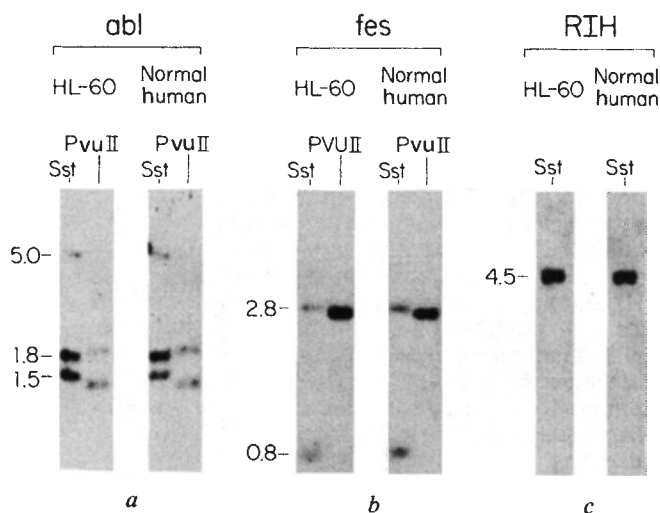


Fig. 2 Absence of amplification of non-*myc* sequences in HL-60 DNA. DNA samples from HL-60 cells and from PHA-stimulated normal human lymphocytes were digested with the designated enzymes, electrophoresed in 1.2% agarose (10 µg DNA per lane), and Southern blot hybridized to the indicated ³²P-labelled probes in conditions described in Fig. 1 legend. The *abl* probe contains the transforming sequences of Abelson leukaemia virus⁴. The *fes* probe contains the transforming sequences of the feline sarcoma virus⁶. The RIH probe is a 0.5-kb clone containing flanking sequences between the human γ and δ globin genes²⁹.

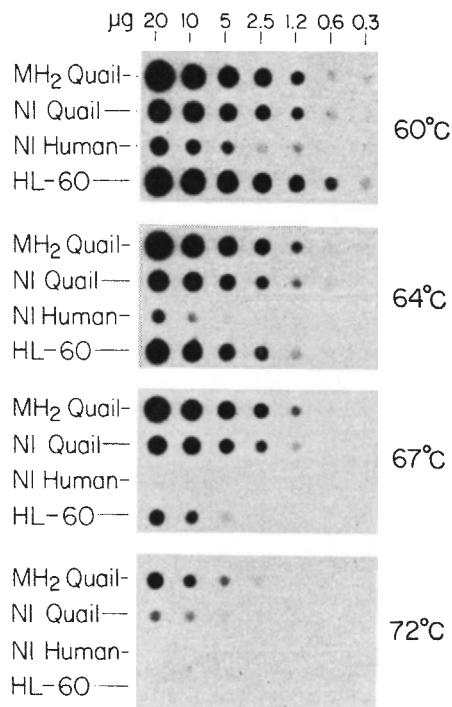


Fig. 3 Melting points of HL-60, normal human and avian DNA *c-myc* hybrids utilizing 'dot-blot' analysis. DNA samples obtained from HL-60 cells, PHA-stimulated human lymphocytes, quail embryo fibroblasts and quail embryo fibroblasts non-productively transformed with the MH₂ virus were treated with RNase A (Sigma) at 50 µg ml⁻¹ for 20 min at 37 °C and then serially two fold diluted followed by denaturation in 1 M ammonium acetate and 0.2 NaOH as previously described²⁰. The indicated quantities of DNA were then applied to a nitrocellulose filter, previously soaked in 1 M ammonium acetate, using a 'Hybri-Dot' manifold (Bethesda Research Laboratories). The filter was then hybridized to the *c-myc* probe, in conditions described in Fig. 1 legend. Following hybridization, the filter was washed in 0.1% SDS, 0.1 × SSC at 50 °C for 20 min, and then exposed for 24 h to XAR-5 film (Kodak) at -70 °C. Following this initial exposure, the filter on consecutive days was washed for 20 min in 0.1% SDS, 0.1 × SSC at progressively higher temperatures as indicated. After each wash, the filter was reexposed for 24 h to XAR-5 film.

with other enzymes, including *Kpn*, *EcoRI* and *HindIII*, although in every case the intensity of hybridization to the HL-60 DNA is much greater (blots not shown). As some of these enzymes presumably cut in sequences flanking, as well as within the human *myc* gene, the identical restriction patterns suggest that the *myc*-containing chromosomal unit amplified in HL-60 may be relatively large. This has been previously noted with amplified CAD²¹ and DHFR²² genes, where the amplified unit seems to be ~500–1,000 kilobases (kb). Thus, as in the examples of CAD and DHFR, it may be difficult to determine by restriction enzyme analysis of HL-60 cellular DNA whether *myc* amplification in HL-60 is tandem or non-tandem.

The *myc* amplification in HL-60 is not a result of hyperdiploidy of the *myc*-containing chromosome because this cell line is hypodiploid (modal chromosome number of 45) and exhibits no hyperdiploidy of individual chromosomes²³. However, we have noted that some HL-60 metaphases contain double minutes and we are now attempting to determine whether these chromosome structures contain amplified *myc*-related sequences.

Hybridization of HL-60 DNA to other 'oncogene' probes derived from the Abelson leukaemia virus⁴ and the feline sarcoma virus⁶ gives a pattern and intensity of hybridization similar to those of normal human DNA (Fig. 2a, b). Moreover, the

c-myc probe, when hybridized to endonuclease digests of DNA from the human myeloid leukaemia cell lines K-562¹⁴ and KG-1¹⁵ as well as to digests from several fresh leukaemia cells, exhibits a pattern and intensity of hybridization similar to those of normal human DNA (blots not shown). This preliminary screen suggests that amplification of endogenous human *c-myc*-related sequences may be an unusual phenomenon in both cultured and fresh human leukaemia cells.

Current speculations on the origin of cancer suggest that malignant transformation may result from the enhanced expression of certain normal cellular genes^{10,12,13}. Indeed, in the case of avian retrovirus-induced bursal lymphomas, increased amounts of *c-myc* RNA as well as amplification of *myc*-related chromosomal DNA sequences have been reported^{12,24}. While the levels of *myc*-related RNA in HL-60 cells and other normal or leukaemic human cells have not been rigorously defined, our own unpublished observations (in collaboration with Paul Neiman) and the results of others³⁰ have revealed relatively high levels of *myc*-related RNA sequences in HL-60. In addition, high levels of *myc*-related RNA have been observed in other leukaemic cells in which the *myc* gene is not amplified (S. Astrin and P. Neiman, personal communications). Thus, these observations may indicate that increased expression of this gene may follow at least two pathways, one involving amplification of *c-myc* DNA (under no obvious selective pressure), and another resulting from increased transcription or stabilization of transcription products from one copy of this gene. It is also possible that the amplification of *c-myc* observed in HL-60 is a normal developmental process in granulocytic differentiation, and that HL-60 represents a specific maturational compartment of these cells, arrested in this state²⁵.

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Sequences on the human Y chromosome homologous to the autosomal gene for argininosuccinate synthetase

S. P. Daiger*, R. S. Wildin & T.-S. Su

Department of Pediatrics, Baylor College of Medicine, Houston, Texas 77030, USA

We have used a probe (pAS-1) to the human urea cycle enzyme argininosuccinate synthetase (AS) to detect individual variation in restriction enzyme digests of human DNA from lymphocytes, fibroblasts and transformed cell lines (including HeLa). pAS-1 is a 1.55 kilobase (kb) sequence of cDNA from poly(A)⁺ RNA cloned in pBR322; it represents more than 90% of the AS message¹. At least 15 genomic fragments are detected with pAS-1 in DNA digested with restriction enzymes recognizing 6-base sequences². This multiplicity of fragments is explained in part by the observation that AS-like sequences are found on more than seven human chromosomes³. We report here population data and gene dosage experiments demonstrating an AS-like sequence on the human Y chromosome and confirming an AS-like sequence on the human X chromosome.

The pAS-1 probe was isolated by plasmid-selected mRNA translation and immunoprecipitation from a bank of cloned human cDNA. The cDNA recombinants were prepared in pBR322 plasmid using sucrose gradient-enriched mRNA from a canavine-resistant colony of the human cell line RPMI 2650. The translation product of mRNA selected by pAS-1 has the immunological properties, molecular weight and substrate specificity of human AS¹.

Human genomic DNA produces more than 15 AS-like fragments when digested with restriction endonucleases recognizing 6-base sequences and more than 25 fragments when digested with 4-base enzymes (Figs 1, 2; Table 1). Restriction analysis with pAS-1 of human-hamster cell lines containing one or two human chromosomes demonstrates that AS-like sequences are found on several human chromosomes including chromosomes 6, 9 and X (ref. 3). (The human AS structural gene is tentatively assigned^{4,5} to 9q34.) The multiplicity of separable DNA fragments and the wide chromosomal dispersion of AS-like sequences makes pAS-1 an excellent probe for restriction site variation in human populations.

In a population survey of restriction site variation, we isolated DNA from more than 50 individuals, digested the DNAs with a panel of six restriction enzymes and probed with pAS-1 following agarose gel electrophoresis and Southern transfer (see Fig. 1 legend for details). DNA was from peripheral blood lymphocytes ($n = 37$), fibroblasts (12), lymphoblasts (4) and transformed cell lines (RPMI 2650 and HeLa). Considerable population variation was detected but this was independent of the type of cell from which the DNA was isolated. Four enzymes, *Bam*HI, *Hind*II, *Hind*III and *Msp*I, revealed independent, high-frequency polymorphic variation in restriction fragments from more than one human autosome. This variation is described elsewhere (S.P.D., R.S.W., N. Hoffman and T.-S.S., in preparation).

A more striking type of variation is apparent in digests with *Bam*HI, *Eco*RI, *Kpn*I and *Msp*I: one or two fragments are always present in males and always absent from females (Table 1, Fig. 1). *Bam*HI, *Eco*RI and *Kpn*I (6-base enzymes) produce one male-specific fragment and *Msp*I (a 4-base enzyme) produces two such fragments. We have observed male-only transmission of these fragments in several three-generation families. Male-specific fragments were also observed in chimpanzees, comparing two male and two female DNAs hybridized with pAS-1.

The most probable explanation for this sexual dimorphism is the presence of an AS-like sequence on the Y chromosome. To confirm this possibility we compared restriction digests of DNA from normal males and from an adult male with an XYY karyotype. (Peripheral blood lymphocytes were used for both DNA and karyotype determination. All of ten cells typed were XYY [Cytogenetics Laboratory, Baylor College of Medicine].) Table 2 shows mean-normal microdensitometry values of the autoradiographs produced in this and related experiments. For the *Bam*HI digest the only significant difference between the normal and XYY male is that the putative Y band (2.45 kb) is nearly twice as dense in the latter, suggesting that roughly twice the normal amount of DNA is present. The male-specific fragments produced by other enzymes (Table 3) are also significantly denser in the XYY DNA. One XX male fibroblast line tested (GM 2626, Human Genetic Mutant Cell Repository, Camden, New Jersey) did not produce a male-specific fragment.

There is one difficulty with this hypothesis: two enzymes, *Hind*III and *Pst*I, produce no apparent male-specific fragment. Double digests with *Hind*III plus *Pst*I also show no difference. We have since tested a few DNAs with four additional enzymes. Two of these enzymes, *Bgl*II and *Sst*I, do produce male-specific fragments and two, *Bcl*I and *Hind*II, apparently do not. The most likely explanation for this discrepancy is that male-specific fragments are present in these digests but are obscured by the multiplicity of other fragments. In the typical X ray picture, 20% of the region from 1.0 to 50 kb contains bands that would obscure additional nearby bands unless they are unusually dense. In general, the putative Y bands are relatively less dense

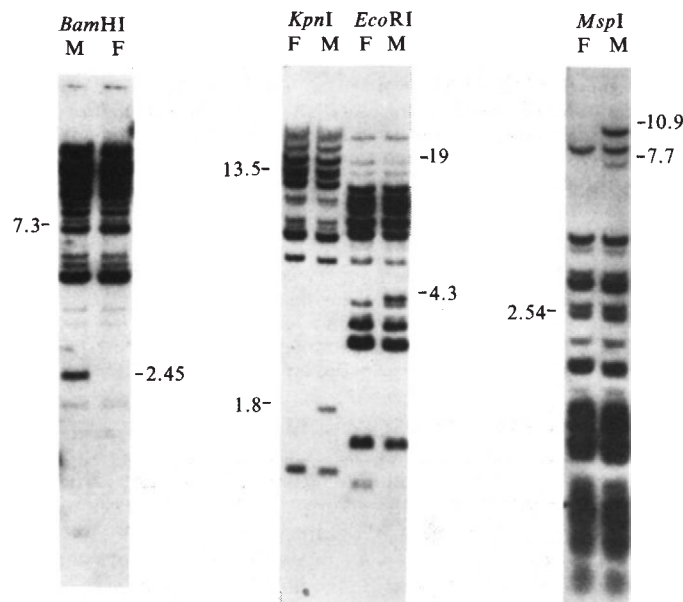


Fig. 1 Human genomic DNA digests probed with ³²P-pAS-1: enzymes producing male-specific fragments. DNA was prepared from 30 cm³ of EDTA-whole blood by recovery of white blood cells from ammonium chloride/ammonium bicarbonate and digestion at 37 °C for 18 h with proteinase K and 0.1% SDS in 50 mM Tris buffer, pH 7.8. DNA was extracted twice with buffer-saturated phenol and precipitated in ethanol, resuspended and digested 1.5 h at 37 °C with RNase A, re-extracted with phenol and dialysed extensively against 10 mM Tris, 0.1 mM EDTA, pH 7.6 (ref. 17). Digestions were 5 µg DNA with 10–40 U restriction enzyme at 37 °C for 6 h. DNA was separated in 0.8% agarose gels, blotted onto nitrocellulose by Southern transfer and hybridized at 65 °C for 36 h with 8 × 10⁵ d.p.m. per lane of pAS-1 (plasmid insert only) labelled with ³²P by nick-translation^{18,19}. Hybridized nitrocellulose was washed at 65 °C with 0.1% SDS and decreasing amounts of sodium chloride/sodium citrate (2 × SSC to 0.5 × SSC). Autoradiography was for 4 days at –70 °C on Kodak SB-5 X-ray film using a DuPont Cronex enhancing screen. Higher-stringency conditions (70 °C, 0.1 × SSC) also produce a faint Y-chromosome fragment. F, female; M, male. Labelled bands are X- and Y-chromosome fragments (in kb); see Table 3.

* Present address: Center for Demographic and Population Genetics, University of Texas Health Science Center, PO Box 20334, Houston, Texas 77025, USA.

Table 1 Number of individuals with male-specific fragments detected with pAS-1

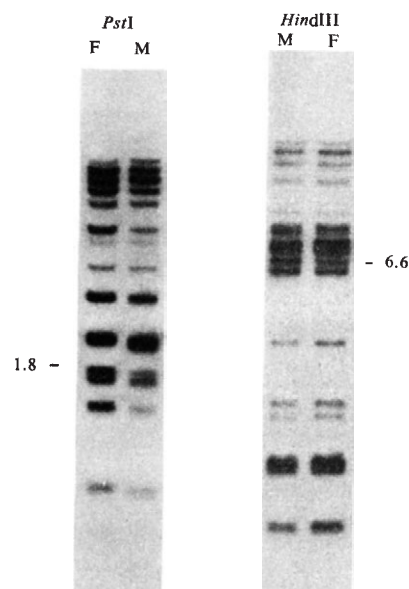
Enzyme	<i>Bam</i> HI		<i>Eco</i> RI		<i>Kpn</i> I		<i>Msp</i> I		<i>Hind</i> III	<i>Pst</i> I
Total no. fragments observed	17		18		16		25		19	16
Male-specific fragments	+	-	+	-	+	-	+	-	None	None
Females	0	20	0	17	0	14	0	17	20	15
Males	30	0	23	0	16	0	18	0	31	22

+, Fragment present; -, fragment absent; none, no male-specific variation observed.

than other bands in the same lane so it is unlikely that microdensitometry alone would detect a male-specific band (Fig. 1; Table 2). (Such density differences may reflect differences in the extent of homology between particular fragments and pAS-1 or differences in the length of homologous sequences.) As no other known biological mechanism would account for these male-specific fragments and since it is certain that some fragments will occasionally obscure other fragments, we believe that all enzymes produce a Y-chromosome fragment containing an AS-like sequence but that these fragments are not always detectable by our methods.

Microdensitometry reveals another type of sexual dimorphism in genomic digests probed with pAS-1: one or two fragments are always denser in females than in males (Table 2). This is consistent with the assignment of an AS-like sequence to the human X chromosome using somatic cell hybridization³. Confirmation by microdensitometry is provided by DNA from a human XO (Turner's syndrome) fibroblast line (GM 857) and an XXXX fibroblast line (GM 1415). The putative X fragments are of equal density in males and in the XO DNA, significantly denser in normal females and most dense in XXXX DNA (Table 2).

Note that although the density ratio of the *Bam*HI Y fragment (2.45 kb), comparing normal male with XYY male DNA, is ~1:2 as expected, the ratio of the X fragment (7.3 kb), comparing normal male with normal female with XXXX female DNA, is 1:1.6:1.8, not the expected 1:2:4. (The XXXX karyotype was confirmed by Barr body determination on cells of the same passage as those used for DNA preparation.) The X-fragment differences, though, are statistically significant and in the correct direction. Conversely, the density of autosomal fragments do not differ between males and females. With such complex

**Fig. 2** Human genomic DNA digests probed with ³²P-pAS-1; enzymes failing to produce male-specific fragments. Methods as for Fig. 1. Labelled bands are X-chromosome fragments (in kb); see Table 3. M, male; F, female.

patterns it is possible that an autosomal fragment underlies the X fragment in *Bam*HI digests; this is the most likely explanation for the discrepancy in X-fragment ratios. Because DNA transfer, hybridization and autoradiography are generally non-linear processes, determination of absolute DNA concentration by microdensitometry is not reliable. In this application, though, with identical internal standards (autosomal fragments) and a sufficient number of controls, microdensitometry is very effective at detecting relative differences in fragment copy number.

Table 3 lists the molecular weights (in kb) of the Y-chromosome and X-chromosome fragments detected in genomic digests and by microdensitometry.

Although Y-chromosome DNA represents ~1% of the total human genome, very few single gene traits have been mapped to this chromosome. 'Hairy ears' has been ascribed to the Y but this assignment is controversial^{6,7}. A 'testis-determining factor' is most certainly on the Y and is associated with the Y histocompatibility antigen (H-Y)⁸. The structural locus for the

Table 2 Mean-normal microdensitometry data

<i>Bam</i> HI						<i>Eco</i> RI			<i>Hind</i> III		
kb	Female <i>n</i> = 7	Male <i>n</i> = 13	XYY 3*	XO 1	XXXX 1	kb	Female <i>n</i> = 3	Male <i>n</i> = 4	kb	Female <i>n</i> = 4	Male <i>n</i> = 10
50	4.9±1.2	4.8±0.8	4.7±0.2	4.7	3.8	31	4.3±1.0	5.0±0.3	32	3.8±0.5	3.8±1.0
30	8.6±1.2	8.7±0.8	6.5±0.7	7.7	6.6	<u>19</u>	6.2±0.8	<u>3.4±0.5</u>	27	5.7±0.4	5.7±0.5
18	11.2±1.2	10.6±0.9	10.7±0.7	10.2	11.8	<u>15</u>	<u>5.1±0.8</u>	<u>4.6±0.4</u>	20	4.5±0.8	4.6±0.6
15	11.5±1.3	11.4±1.5	12.4±0.7	11.0	12.7	11	8.7±0.1	8.0±0.3	16	3.9±0.6	3.9±0.7
13	9.1±0.8	9.7±1.1	10.7±0.1	9.9	10.8	10.5	9.8±0.1	9.4±0.8	11	2.5±0.3	2.2±0.4
11	9.4±1.1	9.8±0.9	10.2±0.5	9.9	9.4	9.5	10.5±0.2	10.2±0.8	9.5	3.2±0.3	2.9±0.8
10.8	9.9±1.2	10.6±1.2	11.1±0.3	10.5	9.9	9.0	9.1±0.9	9.0±1.0	8.6	5.8±0.8	6.1±1.0
10.1	6.1±0.9	5.7±0.7	6.4±0.9	7.5	6.6	7.7	12.0±0.6	12.0±1.2	8.2	5.8±0.6	6.0±1.0
8.9	6.1±1.3	7.3±1.4	7.8±0.3	7.7	6.1	6.9	7.7±0.3	7.6±0.2	7.3	10.2±0.3	10.9±1.1
8.1	4.5±2.1	4.3±2.2	3.3±0.2	5.0	4.7	6.2	1.1±0.2	1.5±0.4	7.2	10.1±0.4	10.3±1.4
<u>7.3</u>	<u>9.9±1.4</u>	<u>6.3±0.7</u>	<u>6.5±0.6</u>	<u>6.4</u>	<u>11.3</u>	5.6	1.7±0.0	1.9±0.6	<u>6.6</u>	<u>9.0±0.5</u>	<u>7.3±0.7</u>
5.9	3.2±0.7	2.7±0.7	2.0±0.3	3.9	2.8	<u>4.3</u>	<u>0.0</u>	<u>3.7±0.9</u>	<u>6.1</u>	<u>7.5±0.2</u>	<u>7.2±0.5</u>
5.5	3.1±0.6	2.8±0.7	1.7±0.2	3.6	3.3	4.0	1.6±0.1	2.9±0.3	3.9	3.9±1.2	4.0±3.0
3.8	1.6±0.5	1.3±0.8	0.6±0.2	1.9	0.5	3.7	2.0±0.1	2.3±0.5	2.7	2.1±1.6	2.3±2.3
<u>2.45</u>	<u>0.0</u>	<u>2.9±1.2</u>	<u>5.0±1.0</u>	<u>0.0</u>	<u>0.0</u>	3.4	5.2±0.2	5.1±0.7	2.5	2.0±0.5	2.1±0.5
						3.0	9.9±0.2	9.0±0.2	2.1	7.1±0.3	7.2±0.5
						1.6	4.0±0.2	4.0±0.6	1.9	7.0±0.2	7.3±0.6
									1.4	4.9±0.3	5.2±0.9

Autoradiographs were scanned with a Helena Laboratories Microdensitometer. Values were normalized by dividing each peak height by the sum of all peaks in a given lane. Data storage and analysis were done on an Apple II microcomputer. Values are arithmetic mean ± 1 s.d. Putative X and Y fragments are underlined. There is an autosomal polymorphism of the *Bam*HI 8.1 kb fragment and of the *Hind*III 2.7 and 3.9 kb fragments. All *Bam*HI individuals in this table are 8.1+; all *Hind*III individuals are 3.9+/2.7+. Some very faint fragments are not included.

* Replicates.

Table 3 Summary of molecular weights (in kb) of sex-specific DNA fragments detected with pAS-1

	<i>Bam</i> HI	<i>Eco</i> RI	<i>Hind</i> III	<i>Kpn</i> I	<i>Msp</i> I	<i>Pst</i> I
Y chromosome	2.45	4.3	—	1.8	10.9, 7.7	—
X chromosome	7.3	19	6.6	13.5	2.54	1.8

Assignment of Y-chromosome fragments is based on male-specific bands detected in genomic digests; —, none detected. Assignment of X-chromosome fragments is based on microdensitometry and human-hamster hybrid cell lines.

H-Y antigen itself, though, may be on another chromosome with regulatory loci on the X and Y⁹. Genes controlling embryonic gonadal differentiation, spermatogenesis and height have also been assigned to the Y¹⁰; these may or may not be independent of the other factors. At best it is reasonable to ascribe only four or five mendelian traits to the Y chromosome.

An explanation for the disparity between the amount of DNA present on the Y and the paucity of convincing gene assignments is that at least 50% of the Y is non-functional heterochromatin. There is considerable cytological variation in human heterochromatin, usually resulting in heritable differences in the size of the Y¹¹. At the DNA level at least two male-specific mid-repeat sequences are detectable with *Hae*III (or *BSu*) digestion^{12,13}. The heterochromatin and mid-repeat sequences are present on the 'inactive' long arm of the Y, whereas genes involved in sexual differentiation map to the short arm apparently. With the exception of heterochromatin differences (and hairy ears possibly), no polymorphic variation in either Y-chromosome DNA or genes has been reported in humans.

Our observation of a Y-chromosome restriction fragment containing an AS-like sequence in humans may provide a unique tool for probing Y-chromosome organization and function. It is highly probable that polymorphic restriction site variation will be detected with at least one of the dozens of restriction enzymes currently in use. These considerations also apply to the AS-like sequence on the X chromosome, though in this case excellent polymorphic markers are already known and many DNA probes are available¹⁴.

The relationship between the AS structural locus and AS-like sequences on the X and Y chromosomes is not yet known in detail. The sequences are short relative to the structural locus but they contain sequences homologous to both 3' and 5' portions of pAS-1 (ref. 3). This arrangement is compatible with pseudogenes that lack introns^{15,16}. The extraordinary dispersion of the AS-like sequence to both X and Y chromosomes and to several autosomes is unprecedented and intriguing.

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Initiator tRNAs from archaebacteria show common unique sequence characteristics

Yoshiyuki Kuchino, Makoto Ihara, Yuko Yabusaki & Susumu Nishimura

Biology Division, National Cancer Center Research Institute, Tsukiji 5-1-1, Chuo-ku, Tokyo 104, Japan

Archaebacteria have recently become of interest in studies on the phylogeny of organisms, for they are quite different from true bacteria as well as from eukaryotes and are thought to be derived from a separate primary line of descents from true bacteria¹. This conclusion is based mainly on nucleotide sequence analysis of 16S ribosomal RNA by Woese and his co-workers, and other genotypic and phenotypic analysis of archaebacteria. To obtain more information on the phylogeny of archaebacteria, we examined the nucleotide sequences of their initiator tRNAs, because much information is available on initiator tRNAs of various organisms, and distinct differences have been found between the sequences of initiator tRNAs of prokaryotes and eukaryotes². We have now determined the nucleotide sequences of initiator tRNAs from *Sulfolobus acidocaldarius*, *Halococcus morrhuae* and *Thermoplasma acidophilum*, and have found that they have a common unique structural feature that differs from both true bacterial initiator tRNAs and eukaryotic initiator tRNAs.

Initiator tRNAs from *S. acidocaldarius*, *H. morrhuae* and *T. acidophilum* were purified from unfractionated tRNA by successive column chromatographies of DEAE-Sephadex A-50, BD-cellulose and RPC-5 (refs. 3,4). Final purification of these tRNAs was achieved by two-dimensional polyacrylamide gel electrophoresis^{5,6}. Heterologous charging of methionine by *Escherichia coli* aminoacyl-tRNA synthetase was adapted to pick up initiator tRNA species during purification⁷. In the case of *T. acidophilum*, two methionine tRNAs were detected when fractionated by BD-cellulose column chromatography (pH 7.5). The formylation reaction with *E. coli* enzyme was used to determine which species was initiator; only one species was formylated, and the other was found to be methionine tRNA for elongation⁸.

The total primary sequences of the three initiator tRNAs were obtained by post-labelling procedures as described previously^{3,6}, and the sequences, arranged in cloverleaf form, are shown in Fig. 1. Eukaryotic initiator tRNAs are distinct from prokaryotic initiator tRNAs in having A₁, t⁶A₃₇, A₅₄, G₅₇, A₆₀ and U₇₂ in place of C₁, A₃₇, T₅₄ (U or uridine derivatives), A₅₇, U₆₀ and A₇₂ (ref. 2). These characteristics have been found in all prokaryotic and eukaryotic (cytoplasmic unicellular and multicellular) initiator tRNAs so far sequenced. By contrast, the three archaebacteria initiator tRNAs all contained A₁, A₃₇, uridine derivative₅₄, (N₄)₅₇ (adenine derivative), U₆₀ and U₇₂. In other words, these particular residues in archaebacteria initiator tRNAs are not the same as those of either prokaryotic initiator tRNAs or eukaryotic initiator tRNAs. These data of archaebacteria initiator tRNAs clearly indicate that archaebacteria are unique prokaryotes distinct from true bacteria. This is further supported by the recent sequence data on 5S RNA⁹ and ribosomal A protein¹⁰.

It is also noteworthy that among archaebacteria initiator tRNAs, *S. acidocaldarius* initiator tRNA shows remarkable resemblance to yeast initiator tRNA; the two tRNAs have almost identical sequences in the CCA-stem, anticodon-stem

Table 1 Sequence homology of initiator tRNAs from archaeobacteria, *E. coli*, yeast, vertebrate and wheat germ

	<i>E. coli</i>	Yeast	Vertebrate	Wheat germ	<i>Thermoplasma</i>	<i>Halococcus</i>	<i>Sulfolobus</i>
<i>E. coli</i>	—	72	71	67	78	71	72
Yeast	—	—	76	73	70	62	82
Vertebrate	—	—	—	87	76	64	74
Wheat germ	—	—	—	—	66	62	71
<i>Thermoplasma</i>	—	—	—	—	—	82	79
<i>Halococcus</i>	—	—	—	—	—	—	72
<i>Sulfolobus</i>	—	—	—	—	—	—	—

D-loops of different chain lengths were arranged to have maximal homology. Unknown modified nucleosides were counted as not having homology. Values are percentage homologies of sequences of initiator tRNAs.

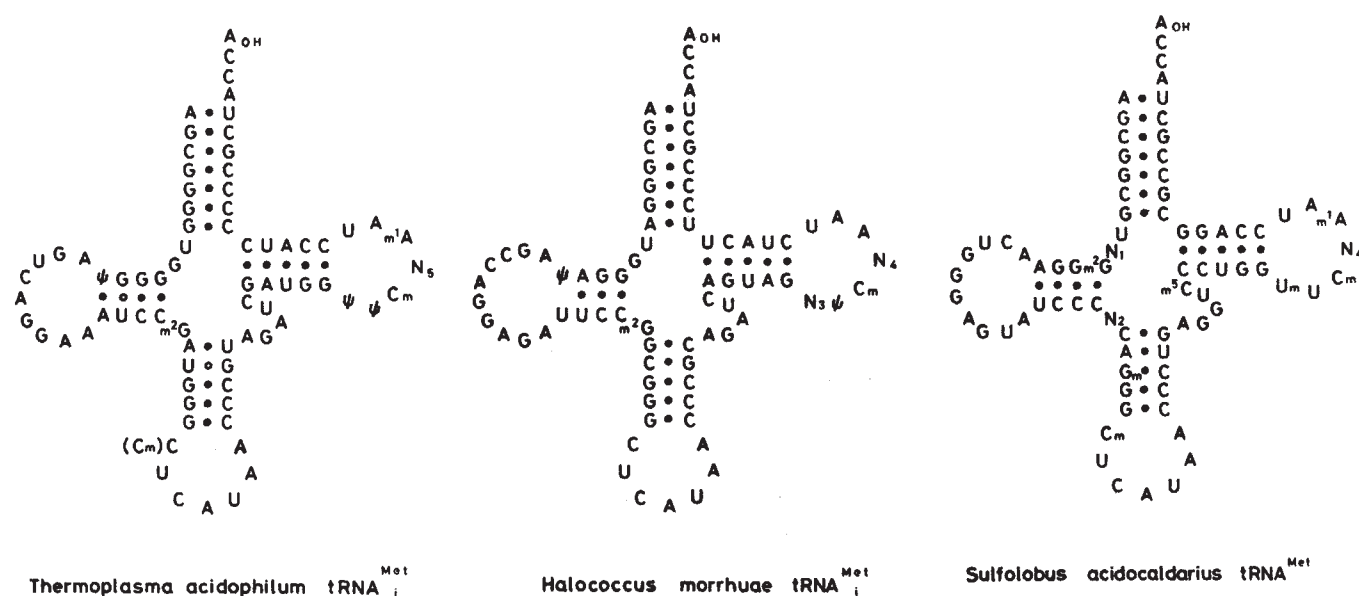


Fig. 1 Nucleotide sequence of initiator tRNAs from *S. acidocaldarius*, *H. morrhuae* and *T. acidophilum* in cloverleaf form. N_3 was recently characterized as 1-methylpseudouridine¹¹. N_2 , N_4 and N_5 are presumably m^2Gm , m^1I and I , respectively (Z. Yamaizumi, Y.K., M.I., W. Zillig and S.N., unpublished results). N_1 is an unidentified modified nucleoside. R. Gupta and C. R. Woese reported (personal communication) that *H. volcani* initiator tRNA contains triphosphate at the 5' end. As we determined the sequences of the tRNAs by the post-labelling technique, we do not know whether they have triphosphate or monophosphate groups at the 5' end.

and anticodon-loop², and the overall homology of *S. acidocaldarius* initiator tRNA to yeast initiator tRNA is 82% while its homology to *E. coli* initiator tRNA is only 72% (Table 1)². By contrast, *H. morrhuae* and *T. acidophilum* initiator tRNAs do not resemble yeast initiator tRNA, or any other eukaryotic initiator tRNAs (Table 1).

All three archaeobacteria initiator tRNAs contain two to four methylated nucleosides in the TΨC-loop. In addition, *S. acidocaldarius* initiator tRNA contains three more 2'-O-methylated nucleosides in other regions. This characteristic is probably related to the tolerance of these tRNAs to extreme growth conditions.

A marked similarity among the three archaeobacterial initiator tRNAs is the identical sequence of five stretches of base pairs in the CCA-stem, although the overall homology between them is not substantially high (Table 1). Fox and his co-workers reported that archaeobacteria can be classified into several major subclusters¹. Judging the phylogeny from 16S rRNA data, *S. acidocaldarius* seems to have separated first from *H. morrhuae* and *T. acidophilum*. The sequence data on initiator tRNAs reported have also suggested that *S. acidocaldarius* is distinct from *H. morrhuae* and *T. acidophilum*. It will obviously be interesting to compare sequences of initiator tRNAs from other archaeobacteria with 16S rRNA data, and work on this line is in progress. The nucleotide sequences of initiator tRNAs from *Halobacterium cutirubrum* and *Halobacterium volcani* have recently been determined by U. L. RajBhandary and S. T.

Bayley and by R. Gupta and C. R. Woese (personal communication), respectively. These sequences are almost the same as that of *H. morrhuae* except for a few base alterations in stem regions and thus are consistent with the above conclusion.

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Mechanism of C-terminal amide formation by pituitary enzymes

A. F. Bradbury, M. D. A. Finnie* & D. G. Smyth

National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, UK

* Department of Mass Spectrometry, St Bartholomew's Hospital, London EC1A 7BE, UK

The amino acid sequences of peptide hormones that terminate in an α -amide group seem usually to be followed by glycine in the sequence of their precursor molecules. Thus it has long been known that the sequence of α -melanotropin, which terminates in Lys-Pro-Val-CONH₂ (ref. 1), occurs adjacent to glycine in the sequence of corticotropin² and more recently it has been shown that the peptide hormone melittin, which terminates in glutamine amide³, is synthesized as a prohormone that terminates in glutamyl-glycine⁴. A similar relationship is suggested by comparison of the primary structures of adipokinetic hormone⁵ and red pigment-concentrating hormone⁶. These observations indicate that biosynthesis of the C-terminal amide may involve the action of a specific enzyme which has a requirement for C-terminal glycine. We present here direct evidence that porcine pituitary contains an enzyme with the ability to convert peptides that terminate in glycine to the corresponding des-glycine peptide amide. We have investigated the substrate specificity of the enzyme and with the aid of isotopic labelling have demonstrated that its mechanism of action involves dehydrogenation and hydrolysis of the glycine-containing substrate.

An assay system for detecting and estimating amidating activity in pituitary was obtained by examining the ability of enzyme preparations to convert the synthetic tripeptide D-tyrosylvalylglycine to the corresponding dipeptide amide D-tyrosylvaline amide. The sequence Val-Gly was selected because it seemed likely to contain the minimum structural elements of corticotropin necessary for formation of the valine amide group present at the C-terminus of α -melanotropin. The D-tyrosine residue conferred stability against degradation by aminopeptidases present in tissue homogenates and provided a site for radioiodination. The ¹²⁵I-labelled tripeptide D-tyrosylvalylglycine was incubated with pituitary homogenates

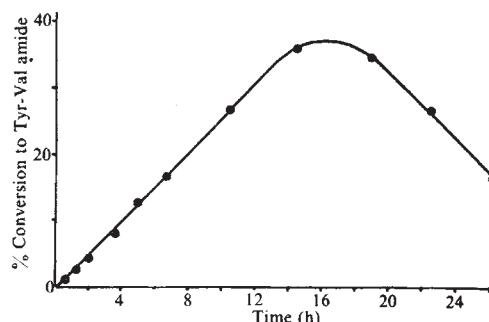


Fig. 1 Formation of D-Tyr-Val amide from D-Tyr-Val-Gly catalysed by an amidating enzyme from porcine pituitary. D-Tyrosylvalylglycine (100 pmol, 10⁶ c.p.m. of ¹²⁵I) was incubated at 37 °C with an enzyme preparation obtained by gel filtration on Sephadex G100, as described in Fig. 3 legend. The incubation was performed with 0.25 ml of the peak fraction in 200 mM sodium chloride, 50 mM sodium phosphate at pH 7. At intervals, 20- μ l aliquots of the reaction mixture were removed, acidified with 10 μ l of 1 M hydrochloric acid and chromatographed on a column (15 $\times$ 1 cm) of SP Sephadex C25 eluted in 200 mM sodium chloride and 50 mM sodium phosphate at pH 5.5. The yield of D-Tyr-Val amide was calculated from the proportion of the total radioactivity that emerged in a peak corresponding to the position of an ¹³¹I-labelled marker peptide. The iodinated tripeptide emerged at 20 ml and the iodinated dipeptide amide at 51 ml.

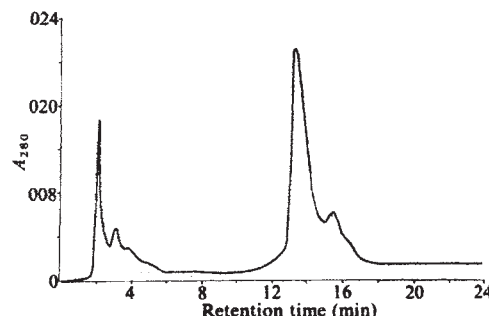


Fig. 2 Isolation of D-Tyr-Val amide produced by incubation of D-Tyr-Val-Gly with amidating enzyme extracted from porcine pituitary. The fraction containing the tritium-labelled dipeptide amide, obtained by ion-exchange chromatography as described in Fig. 1 legend, was concentrated *in vacuo* and submitted to HPLC on a C18 Microbondapak column (25 $\times$ 0.4 cm) eluted in 2 mM hydrochloric acid with a linear gradient (20 min) from 5–15% methanol. Fraction size was 0.5 ml. The D-Tyr-Val amide emerged at 27 ml.

(0.5 g porcine pituitary in 1 ml of phosphate-buffered saline, pH 7.4) or with chromatographed preparations of amidating enzyme obtained from porcine pituitary, and the degree of conversion of the D-Tyr-Val-Gly to D-Tyr-Val-CONH₂ was determined by column chromatography of the radiolabelled peptides on sulphopropyl Sephadex C25. The elution positions of the iodinated tripeptide and dipeptide amide were established with the use of synthetic peptides.

Incubation of D-tyrosylvalylglycine with a preparation of the amidating enzyme obtained by gel filtration on Sephadex G100 led to the progressive formation of D-tyrosylvaline amide (Fig. 1). In the conditions used, the yield of the dipeptide amide reached a maximum value of 38% in 17 h, then slowly declined. Control experiments showed that when synthetic ¹²⁵I-labelled D-tyrosylvaline amide was incubated in the same conditions it was slowly degraded. Thus the formation of the dipeptide amide by the pituitary enzyme is followed by its destruction by other enzymes present in the pituitary extract. The reaction product, D-tyrosylvaline amide, was identified by co-elution with ¹³¹I-labelled synthetic D-Tyr-Val amide; and in further experiments co-chromatography was demonstrated with D-Tyr-³H-valine amide formed from D-Tyr-³H-valylglycine with the corresponding synthetic dipeptide amide. The identity of the D-Tyr-Val-CONH₂ generated by the pituitary enzyme was further confirmed by its isolation in sufficient quantity for amino acid analysis. The incubation was performed using chemical amounts

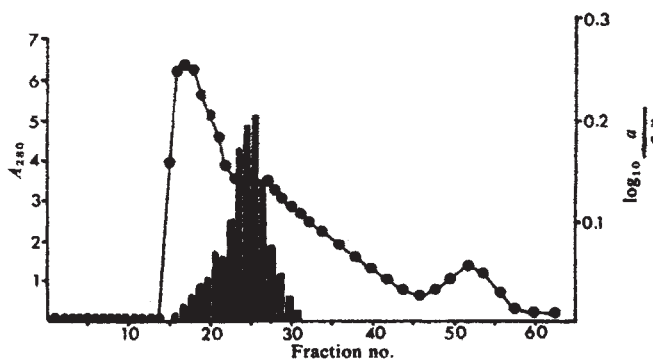


Fig. 3 Purification of amidating enzyme from porcine pituitary by gel filtration on Sephadex G100. The soluble enzyme obtained from the secretory granule fraction of porcine pituitary (50 g) was concentrated by vacuum dialysis and chromatographed on a column (60 $\times$ 1.5 cm) of Sephadex G100 eluted in 200 mM sodium chloride and 50 mM sodium phosphate at pH 7; fraction size was 2.5 ml. The enzyme was assayed by incubating 50- μ l portions of each fraction with ¹²⁵I-D-Tyr-Val-Gly (20 pmol, 2 $\times$ 10⁵ c.p.m.) at pH 7 for 5 h at 37 °C. The yields of dipeptide amide formed were determined as described in Fig. 1 legend.

of D-Tyr-Val-Gly (100 nmol containing 2 μ Ci of 3 H-labelled peptide) in 10 ml of 200 mM sodium chloride, 50 mM sodium phosphate, pH 7.0, at 37°C for 10 h in the presence of enzyme purified on Sephadex G100. The resulting dipeptide amide was isolated in homogeneous form by gel filtration on Sephadex G25 to remove higher molecular weight material, by ion exchange chromatography as in Fig. 1 and by reverse-phase HPLC (Fig. 2). The reaction product (6 nmol) gave an amino acid composition of Tyr, 1.0; Val, 0.9; Gly, <0.1; NH_3 , 1.1 residue.

The amidating enzyme was shown to be present in the secretory granule fraction of porcine pituitary. The granules, prepared from 50 g porcine pituitary by differential centrifugation⁷, were solubilized by repeated sonication in 10 mM sodium phosphate at pH 7.0. The extracted enzyme was concentrated by dialysis under reduced pressure and purified by chromatography on Sephadex G100 (Fig. 3). The amidating activity emerged in a broad fraction, indicating that the minimum apparent molecular weight of the enzyme was ~60,000. Significant amidating activity could also be demonstrated, however, in the fractions corresponding to a molecular weight greater than 100,000 but further gel filtration of the higher molecular weight material gave a similar elution pattern to the initial granule extract, suggesting that the enzyme can undergo reversible aggregation. The yields of dipeptide amide obtained with the purified enzyme were ~40%, which is an order of magnitude greater than could be obtained with unfractionated pituitary homogenates.

To investigate the mechanism of amidation, the reaction was carried out with isotopically labelled tripeptides. D-Tyrosyl-

Table 1 Mass measurements of the ions in the spectra of products formed enzymatically from D-Tyr-Val- ^{14}N -Gly (a) and D-Tyr-Val- ^{15}N -Gly (b)

Nominal mass	Empirical formula	Calculated mass	Measured mass	Error
346(a)	$\text{C}_{11}\text{H}_{10}\text{O}_3\text{N}_3\text{F}_6$	346.0624	346.0612	-0.0012
549(a)	$\text{C}_{20}\text{H}_{16}\text{O}_5\text{N}_3\text{F}_9$	549.0942	549.0954	+0.0012
347(b)	$\text{C}_{11}\text{H}_{10}\text{O}_3^{15}\text{N} \cdot \text{N}_2\text{F}_6$	347.0594	347.0596	+0.0002
550(b)	$\text{C}_{20}\text{H}_{16}\text{O}_5^{15}\text{N} \cdot \text{N}_2\text{F}_9$	550.0912	550.0928	+0.0016

valylglycine was synthesized using both ^{14}N -glycine and ^{15}N -glycine and the corresponding D-tyrosylvaline amides were isolated after incubation with the purified pituitary enzyme. The molecular structures of the products were confirmed by high- and low-resolution mass spectrometric analysis of the trifluoroacetyl derivatives (Fig. 4) prepared by reaction of the peptide amides with trifluoroacetic anhydride in anhydrous conditions⁸. The low resolution mass spectra obtained from the enzymatically derived dipeptide amides were compared with those obtained from the corresponding synthetic dipeptide amides (Fig. 5). Three of the ions obtained showed the single mass unit increment expected from the incorporation of the ^{15}N isotope. High-resolution mass measurements of the molecular ions at 549 and 550 AMU (atomic mass units) and the base peaks of the spectra at 346 and 347 AMU gave unequivocal evidence for the empirical formulae of these compounds (Table 1), proving that the ^{15}N isotope from the glycine residue was retained in the peptide amide. This finding rules

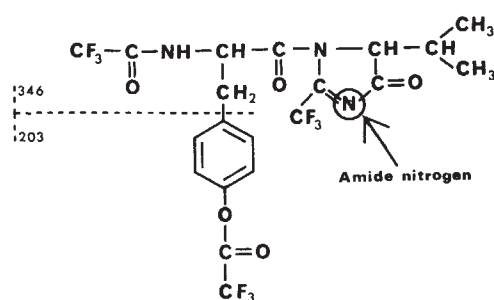


Fig. 4 Structural formula of the trifluoroacetyl derivative tyrosylvaline amide.

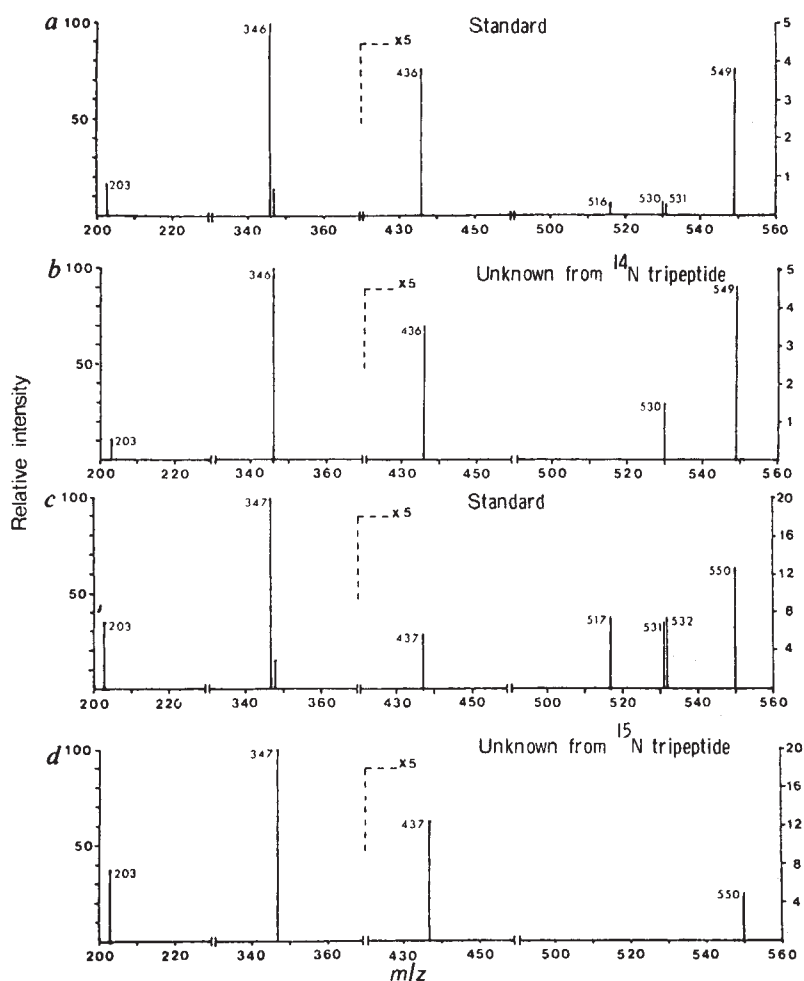
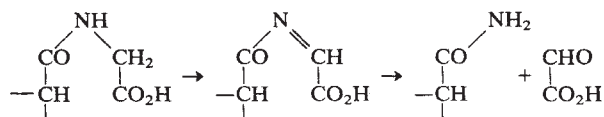


Fig. 5 Normalized partial mass spectra of the trifluoroacetyl derivative of Tyr-Val amide. a, Synthetic D-Tyr-Val ^{14}N -amide. b, D-Tyr-Val amide formed enzymatically from Tyr-Val- ^{14}N -Gly. c, Synthetic D-Tyr-Val ^{15}N -amide. d, D-Tyr-Val amide formed enzymatically from Tyr-Val- ^{15}N -Gly.

out the possibility that the amide group might arise from ammonia, either by direct amidation of the valine carboxyl group⁹ or by transamidation of the valylglycine peptide bond¹⁰. The data obtained support a mechanism involving removal of hydrogen from the C-terminal glycine and spontaneous hydrolysis of the resulting imino linkage:



Consistent with this oxidative mechanism, it was found that when the incubation was performed with D-Tyr-Val-Gly synthesized with ¹⁴C-glycine, the isotope was incorporated into glyoxylate. Furthermore, the amidation reaction was not affected by the presence of 1 mM ammonium ion, glutamine or asparagine at similar concentrations. The proposed mechanism is also consistent with previous suggestions that unsaturated peptides may be formed transiently during the biosynthesis of C-terminal amides^{11,12}.

The substrate specificity of the amidating enzyme was examined with a range of synthetic tripeptides. It was found that glycine was a mandatory amino acid in the C-terminal position of the tripeptide D-Tyr-Val-Gly; substrates containing leucine, alanine, sarcosine, lysine or glutamic acid in place of the glycine did not give rise to a detectable amount of dipeptide amide. Thus no amidation would be expected to take place with D-Tyr-Val-COOH as substrate, and none was observed. It can be concluded that the dipeptide carboxylate is not an intermediate in the formation of D-Tyr-Val amide from D-Tyr-Val-Gly. That C-terminal glycine should act as a signal for amidation is consistent with recent reports that the sequence of vasopressin is followed by glycine in the prohormone of vasopressin¹³ and that glycine is present immediately after the C-terminal residue of calcitonin in the sequence of its biosynthetic precursor¹⁴.

Amidation of the tripeptide D-Tyr-Val-Gly was found to take place when the valine in position 2 was replaced by phenylalanine or glycine but not when it was replaced by D-alanine, lysine or aspartic acid. This suggests that the pituitary enzyme can catalyse the formation of the amide group from the precursors of gastrin, cholecystokinin, oxytocin or vasopressin, hormones that terminate in phenylalanine or glycine amide. Furthermore, as the pituitary enzyme can form amides only with neutral amino acids in position 2 of the tripeptide substrates, and all known hormone amides appear to contain a neutral amino acid in the C-terminal position, it is clear that the pituitary enzyme has similar properties to the enzymes involved in the amidation of hormones in other tissues. The amidating enzyme in porcine pituitary may prove to be common to all tissues that biosynthesize the C-terminal carboxamide group.

We thank D. M. Carter for performing high-resolution mass measurements and product spectra. The Mass Spectrometry Unit is supported by grants from the Wellcome Trust.

Effect of an allozyme polymorphism on regulation of cell volume

Thomas J. Hilbish, Lewis E. Deaton
& Richard K. Koehn

Department of Ecology and Evolution, State University of New York, Stony Brook, New York 11794, USA

Biochemical differences have been described among allozymes¹⁻⁴, but the physiological consequences (that is, physiological phenotypes) of such differences have only rarely been demonstrated⁵⁻⁷. Such a relationship is necessary both to the argument that natural selection maintains allozymic diversity in natural populations and for understanding biochemical mechanisms of adaptation⁸. We report here a genotype-dependent difference in the rate of cellular free amino acid accumulation during adjustment to hyperosmotic conditions in the mussel *Mytilus edulis*. The product of the *Lap* locus in *M. edulis* is the lysosomal enzyme aminopeptidase-I (AM-I; E.C.3.4.11.-) which hydrolyses oligopeptides to their constituent amino acids⁹. Total AM-I activity is positively correlated with salinity; a 120% increase in salinity increases AM-I activity twofold¹⁰⁻¹³. Adjustment to hyperosmotic stress in osmoconforming marine bivalves, including *M. edulis*, involves rapid accumulation of cellular free amino acids^{14,15}. The biochemical properties of AM-I, and its activation by salinity changes, suggest that it is important for providing cellular free amino acid pools during adjustment to hyperosmotic stress¹¹. We show that individuals carrying the allele for high catalytic efficiency (*k_{cat}*) accumulated cellular amino acids more rapidly than other genotypes. The difference in accumulation rate was also demonstrated by an interruption of hyperosmotic adjustment, which resulted in genotype-dependent rates of amino acid excretion. These results demonstrate the role of AM-I in the physiological processes that regulate cell volume. Thus, differing catalytic properties of allozymes are manifested as phenotypic differences at the physiological level and provide a mechanism for the known selective mortality of mussels in natural populations⁷.

Allozymes of AM-I have different catalytic properties; genotypes with the *Lap*⁹⁴ allele (in either the homozygous or heterozygous condition) exhibit about 20% greater activity per unit enzyme protein, due to the higher *k_{cat}* of this allele². This difference in specific activity, together with the proposed function of the enzyme in cell volume regulation, dictates that: first, on exposure to hyperosmotic stress, individuals with the *Lap*⁹⁴ allele should accumulate cellular free amino acids at a greater rate. As the cellular response to hypo-osmotic stress consists of the release of amino acids to the haemolymph, some of which

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Table 1 Tissue-specific levels of amino acids (ninhydrin-positive substances) as a function of AM-I genotype

	Adductor	Mantle	Digestive gland
Control	539 ± 15.0 (10)	566 ± 27.8 (10)	439 ± 7.3 (9)
<i>Lap</i> ⁹⁴	681 ± 12.7 (29)	680 ± 21.8 (28)	617 ± 6.4 (29)
Non- <i>Lap</i> ⁹⁴	664 ± 15.8 (11)*	655 ± 25.2 (15)*	570 ± 14.7 (16)†

All measurements were made 70 h after transfer of individuals from 15 p.p.t. to 30 p.p.t. salinity. *Lap*⁹⁴ indicates those genotypes with the allele and non-*Lap*⁹⁴ refers to all other genotypes. All values are mean ± s.e. in nmol per mg dry weight. Sample size is in parentheses.

* Difference between the two non-control groups not significant at *P* < 0.05; one-tailed *t*-test.

† Significant difference (*P* < 0.005).

are ultimately excreted¹⁶, differences among animals in the size of the amino acid pool should result from different rates of amino acid excretion. Therefore, a second prediction is that during adjustment to hyperosmotic stress, transfer to hypo-osmotic conditions will produce a greater rate of excretion of amino acids by *Lap*⁹⁴ individuals.

To test these predictions, animals were acclimated in 15 parts per trillion (p.p.t.) salinity for 2 weeks, then transferred to 30 p.p.t. and the concentration of cellular free amino acids observed during hyperosmotic adjustment. In the digestive gland, there was an increase in ninhydrin-positive substances (NPS; that is, principally free amino acids) of ~160 nmol per mg dry weight. Ten individuals maintained at 15 p.p.t. were taken at each sample period as controls, to demonstrate significant NPS accumulation in high salinity treatments. While the controls were heterogeneous ($P < 0.025$; analysis of variance), the variance was due to an elevated mean of the 48-h sample; variation among controls will not affect the tests for relative differences among genotypes. All control variates were combined to give an overall mean and standard error (Fig. 1). The increase in NPS was clearly genotype-dependent (Fig. 1; Table 1); after 48 h at 30 p.p.t. the differences between *Lap*⁹⁴ and other genotypes approached significance ($P < 0.1$; one-tailed *t*-test) and after 70 h at 30 p.p.t., *Lap*⁹⁴ genotypes had a 24% greater net increase in NPS ($P < 0.005$) than other genotypes. One-tailed comparisons were dictated by the *a priori* prediction of greater NPS accumulation in *Lap*⁹⁴ genotypes because of the higher catalytic efficiency of individuals carrying this allele.

The digestive gland has the greatest AM-I activity of any tissue; mantle and adductor tissues have maximally 50% and 5%, respectively, of the activity found in the digestive gland^{13,17}. There were no significant contributions of genotype to the rates of accumulation of NPS in either mantle or adductor tissues, even after 70 h of hyperosmotic adjustment, although the results suggested greater rates of NPS accumulation for genotypes containing *Lap*⁹⁴ (Table 1). We interpret the lack of genotype-dependent differences in mantle and adductor tissues as being due to the low AM-I activity in these tissues.

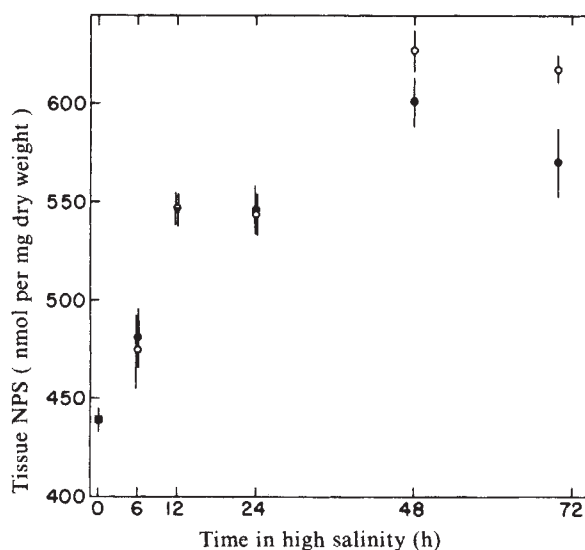


Fig. 1 Accumulation of ninhydrin-positive substances (NPS, nmol per mg dry tissue weight) in digestive gland of *Mytilus edulis* after transfer from 15 p.p.t. to 30 p.p.t. salinity. ○, Genotypes containing *Lap*⁹⁴; ●, genotypes without *Lap*⁹⁴; ■, overall mean for controls. The determination of NPS was modified for small samples¹⁹. *Lap* genotypes were determined by starch gel electrophoresis²⁰.

Table 2 Genotype-specific rates of amino acid excretion during hypo-osmotic stress

	24 h	48 h	70 h
<i>Lap</i> ⁹⁴	304 ± 103 (17)	150 ± 38 (25)	497 ± 75.0 (31)
Non- <i>Lap</i> ⁹⁴	296 ± 78.1 (9)*	183 ± 49.3 (16)*	271 ± 55.5 (15)†

Animals were acclimated to 15 p.p.t. salinity, 15 °C for 2 weeks before transfer to 30 p.p.t. After 24–70 h, animals were returned to 15 p.p.t. for 2 h. They were then placed in 150 ml of 15 p.p.t. filtered (0.45 µm pores) artificial seawater for 2.5 h. The amino acid concentration of duplicate 1-ml samples was determined using the fluorimetric method¹⁸. Results are expressed as nmol of glycine equivalents per g dry weight per h.

* Difference between genotypic groups not significant at $P < 0.05$.

† Significant difference ($P < 0.01$; one-tailed *t*-test) assuming inequality of variances.

In animals returned to 15 p.p.t. salinity after 70 h at 30 p.p.t., amino acid excretion reflected the genotype-dependent accumulation differences (Table 2). Genotypes with the *Lap*⁹⁴ allele showed almost twice the excretion rate of genotypes without the allele. There was a notable concordance between genotype-dependent differences in accumulation during hyperosmotic adjustment and genotype-dependent excretion during hypo-osmotic stress; there were no significant differences in excretion rates until sufficient time had elapsed for significant differences in NPS accumulation to occur (Table 2).

Thus we have demonstrated that known catalytic differences among allozymes have predictable and measurable physiological consequences. In natural situations of diminished or fluctuating salinity, there is a direct ecological 'cost' to possessing the *Lap*⁹⁴ allele. Our results provide a possible mechanism for the demonstrated selective mortality of *Lap*⁹⁴ genotypes among natural populations experiencing differing salinities⁷. In a situation of natural environmental variation, an imbalance between accumulation and excretion of amino acids may lead to differential and excessive loss of nitrogenous resources by *Lap*⁹⁴ genotypes. Specific studies of nitrogen balance in field populations are presently underway.

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ANNOUNCEMENTS

Awards

The eighth Marconi Fellowship for Communications, Science and Technology has been awarded to **Arthur C. Clarke** for his contributions to the invention of the communications satellite. Most of the \$35,000 grant will be used at the proposed Centre for Third World Communication, Energy and Space Technologies, at the University of Moratuwa, near Colombo, Sri Lanka, of which he is chancellor.

The World Academy of Art and Science has awarded the II Stuart Mudd Award to **Professor Leonardo J. Mata** (Institute of Research in Health, University of Costa Rica) for his pioneer work on intestinal microecology of poor and traditional populations of Central America living in different ecosystems.

The trustees of the Lady Tata Memorial Trust have made international awards for research in leukaemia to **H. Abrams** (USA), **H.J. Allen** (UK), **D.W. Anderson** (USA) (renewed awards); **R.J. Baer** (USA), **A.P. Gee** (USA), and **A.R. Venkitaraman** (India) (new awards).

Sir Alastair Pilkington has been awarded the Messel Medal of the Society of Chemical Industry for 1982. The Medal is the premier award of the Society and is awarded every second year.

The Royal Society of Chemistry presented its annual film prize on 8 June to the producers of the German film *Juncture* which describes work at the BASF chemical plant at Ludwigshafen.

The Royal Society of Chemistry has awarded the Meldola Medal and Prize jointly to **Dr D.C. Clary** (UMIST), for contributions to the theory of molecular energy transfer and chemical reactions, and to **Dr A. Dilks** (Xerox Corporation in Rochester, New York), for work on the characterization and plasma modifications of polymer surfaces.

The Meldola Medal, now accompanied by a prize of £100, will next be awarded in 1983 to the chemist who, being a British subject and under 30 years of age at 31 December 1982, shows the most promise as indicated by published chemical work. Applications, by 31 December 1982, to The President, The Royal Society of Chemistry, Burlington House, London W1, UK, the envelope being marked Meldola Medal.

An award of SF5,000 and a silver medal is available every three years, for the best paper or report devoted to significant work pertaining to coal tar products, plant or processes. Full details and conditions from BTIA, 132/135 Sloane Street, London SW1, UK.

The Beilby Awards, which consists of a medal and a prize of £250, are made to British investigators in science in recognition of original work of exceptional merit which has led to advances of practical significance. This work should have involved the development and application of scientific principles in chemical engineering, fuel technology or metallurgy. The awards are intended as an encouragement to younger men and women, normally under the age of 40. Applications, by 31 December 1982, to The Convener of the Administrators, Sir George Beilby Memorial Fund, The Royal Society of Chemistry, Burlington House, London W1, UK.

The W.K. Kellogg Foundation of America is providing \$3 million to enable Oxford University to develop a major centre for continuing education. The Rewley House site in Wellington Square, part of which was acquired and converted into a residential centre for adult students with considerable financial help from the Foundation in 1963, will be redeveloped. Its new conference facilities will include a large lecture theatre, eight fully-equipped seminar rooms, a library and an administrative block. Existing residential accommodation will be upgraded, and catering facilities enlarged.

Appointments

Dr Lewis M. Branscomb has been re-elected chairman and **Dr Mary L. Good** vice chairman of the National Science Board, the governing body of the National Science Foundation.

Dr George Bugliarello (President of the Polytechnic Institute of New York) is the new Council Secretary of the Marconi International Fellowship Council following the retirement of Dr Walter Orr Roberts.

Prof. Alvin M. Kaye, has been named as the first incumbent of the Joseph Moss Chair in Molecular Endocrinology, established at the Weizmann Institute of Science.

Prof. R.J. Berry (University College, London) has been elected president of the Linnean Society for the next three years, succeeding Prof. W.T. Stearn. **Dr Colin Patterson** was elected as editorial secretary and the other officers were re-elected.

The Zoological Society of London has elected **Professor John Phillips** (Director of the Wolfson Institute in the University of Hull) as its next Secretary.

Prof. Janet V. Watson (Geology, Imperial College of Science and Technology) has been elected president of the Geological Society.

Prof. David G. Harnden (Department of Cancer Studies, University of Birmingham) has been appointed Director Designate of the Paterson Laboratories, Christie Hospital and Holt Radium Institute at the University of Manchester, to succeed the present Director, Professor L.G. Lajtha on his retirement in June 1983.

Meetings

1-3 September, **International Hepatitis Workshop**, Stirling (Miss E.K.S. Grant, Nuclear Enterprises Ltd, Sighthill, Edinburgh, UK).

1-3 September, **Annual Symposium Uranium Institute**, London (Mrs C. Scott Barrett, Conference Associates UIS, 34 Stanford Rd, London W8, UK).

1-3 September, **Fast Reactions in Solution**, Leuven (Dr A. Persoons, Dept of Chemistry, University of Leuven, Leuven, Belgium).

1-3 September, **The International Environment and Safety Exhibition and Conference**, Wembley (Labmate Ltd, Sandpit Lane, St Albans, Herts, UK).

3-9 September, **Science and Technology in the Service of Conservation**, Washington DC (IIC American Meeting, 1511 K Street NW, Suite 725, Washington DC 20005, USA).

5-9 September, **Protein Metabolism and Nutrition**, Clermont-Ferrand (4ème SIMNA, INRA-Teix, Laboratoire d'Etude de Metabolisme Azoté, 63110 Beaumont, France).

5-11 September, **World Congress on Medical Physics and Biomedical Engineering**, Hamburg (Hamburg Messr, Congress Organisation, D-2000 Hamburg 36, PO Box 30 23 60, FRG).

5-15 September, **Charles Coulson Summer School in Theoretical Chemistry**, Oxford (Dr P.J. Grout, Theoretical Chemistry Dept, 1 South Parks Rd, Oxford, UK).

6-8 September, **Charged Particles Management of Electrostatic Hazards and Problems**, Southampton (Miss L. Claff, Scientific and Technical Studies, Oyez IBC Ltd, 11-13 Norwich St, London EC4, UK).

6-8 September, **Modern Aspects of Heterocyclic Chemistry**, Sussex (Miss L. Hart, Royal Society of Chemistry, 30 Russell Square, London WC1, UK).

6-9 September, **Distillation**, Geneva (The Center for Professional Advancement, Postbus 19865, 1000 GW Amsterdam, The Netherlands).

6-10 September, **Annual Meeting, British Association for the Advancement of Science**, Liverpool (Ms U. Laver, BAAS, Fortress House, 23 Savile Row, London W1, UK).

6-10 September, **Linear Programming and Operational Planning in the Petroleum Chemical and Process Industries**, Edinburgh (Education Secretary, Institution of Chemical Engineers, 12 Gayfere St, London SW1, UK).

BOOK REVIEWS

The vision of David Marr

Stuart Sutherland

In 1973 David Marr, then 28 years old, moved from Cambridge, England, to Cambridge, Massachusetts; seven years later he died of leukaemia. During this period he formulated a new approach to the theory of vision and published his results in a series of papers of great depth. Much of Marr's originality lay in his insistence that one must have a rigorous theory of how a particular task is performed before proceeding to construct a computer model of how the brain carries it out. *Vision*, posthumously published, presents a survey of his own work and that of the group of exceptionally clever young scientists whom he inspired with his ideas.

Despite his originality, Marr's papers were often flawed. Many were poorly written and unnecessarily opaque. Moreover, each paper tackled a particular aspect of vision and it was sometimes hard to see the connections. Marr's book overcomes both problems. It is written in a lively style and is for the most part lucid and free from technicalities. In addition, it has a clear structure that integrates the different aspects of his research.

Marr believed that there are three main levels of visual processing, at each of which a separate representation of the world is formed. At the lowest level is "the primal sketch", a representation of the retinal image that makes explicit the size and disposition of the intensity changes present. The primal sketch itself contains several levels of representation. At its highest levels it represents boundaries formed by grouping lower level elements. Such a boundary may be formed, for example, by a change in the density or size of a set of circles present in the retinal image. The purpose of the primal sketch is to deliver to higher level processes an explicit description of the boundaries that exist in the image and to enable these processes to determine whether the boundaries found are caused by a change in reflectance, a change in light intensity, an occluding contour or an edge at which two visible surfaces of a body meet.

Much the longest section of Marr's book is devoted to the processes that operate on the primal sketch in order to recover a 3-dimensional representation of the world. Amongst these processes are stereopsis, and those that use shading, texture, occluding contours and various aspects of motion. From these cues the second level of representation; the "2½-D sketch", is constructed: this is a representation of the scene based on viewer-centred coordinates, which makes explicit the slope relative to

Vision: A Computational Investigation into the Human Representation and Processing of Visual Information. By David Marr. Pp.397. ISBN 0-7167-1284-9. (W.H. Freeman: 1982.) £22.50, \$39.95.

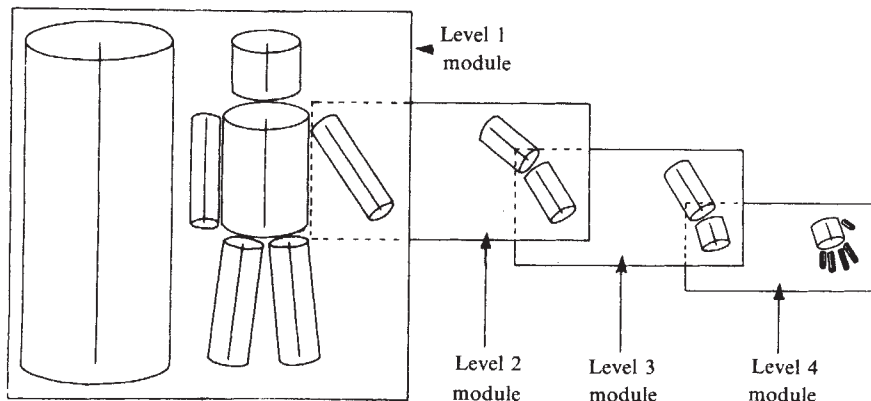
the viewer at each point on all visible surfaces. For the purpose of recognizing objects, a 3-D model, the highest level of representation, is formed from the 2½-D sketch. This model represents objects in object-centred coordinates (based either on the direction of elongation of the object or on its symmetry) and it makes explicit the volumetric structure of the object by representing its parts as generalized cones (the volume swept out by moving a cross section of a particular shape along an axis).

Not only does *Vision* have a clear structure, it also gives an integrated treatment of each stage of processing, based on Marr's philosophy of the nature of explanation in psychology. He distinguishes three levels of explanation. At the highest level is a *computational theory*, that is a theory of how a task can be performed. At the next level is a *representation* and an *algorithm* to achieve that representation. At the lowest level lies the question of how the algorithm is actually implemented in the *hardware* of the system.

Marr's originality and much of the depth of his own thinking stems from his emphasis on the top level type of explanation, which few had previously even considered. The computational theory for a given stage in processing should, according to Marr, specify what is being computed, and why this is a useful thing to compute. It should in addition specify the ways in which the image is

related to the world, that is to say, the constraints on how the image can be interpreted. It is only the existence of these constraints that makes it possible to recover from the image the properties of the scene. Thus, in recovering the shape of a rotating body from successive views of it, it is necessary to assume that the body is rigid, since otherwise there is an infinite number of possible interpretations. The assumption will normally hold true; where it does not, no solution to recovering a body's 3-D shape from its motion will emerge. Again, stereopsis depends on the constraints that in general one and only one point on one retina receives light from the same source as a single point on the other, and that local changes in disparity will be small. Both constraints have exceptions — in parts of the visual field there will be objects or parts of objects that are seen by one eye but not the other, and where one object occludes another there will be sudden changes in disparity. But for the most part the constraints hold, because the world is largely made up of surfaces that are comparatively smooth. Only by using these constraints is it possible to decide which point in the image on one retina corresponds to a given point in the image on the other.

The computational theory of a given task not only constrains the nature of the algorithm, it also constrains the nature of the representation of the results of a given stage of processing. Thus, Marr justifies the primitives used in the primal sketch (for example, segments of lines and edges formed by brightness changes, and higher level boundaries) by noting that it is just this sort of information that is needed to recover at the level of the 2½-D sketch the



Hierarchical organization of a 3-D model: the visual object (here a person) is decomposed into parts, and each part is decomposed into successively finer and finer detail — in this case whole body, arm, forearm and hand. Each part is represented by its central axis and a generalized cone that corresponds to the volumetric shape of its surface. This method of representation preserves gross similarities between objects whilst making explicit variations in detail.

local orientation of surfaces and their reflectance. Again, in deciding how to represent the 3-D model of an object, Marr is guided by a computational theory of the process of recognition. First, the representation has to be accessible, that is, it must be possible to compute it economically from the image. Second, it should have a wide scope in that it must be possible to represent any object that can be recognized. Third, it must provide a unique description of a given object, otherwise different descriptions would be formed on different occasions and hence recognition would fail. Fourth, the representation should reflect the similarity between similar objects, but should also make explicit small differences: we can both see the similarity between different people and can also recognize individuals. Marr's method of representing 3-D objects meets all these constraints. Thus, the last constraint is satisfied by organizing the representation hierarchically so that the coarse shape of an object is represented in one module with other modules representing successively finer and finer detail (see diagram p.693).

There is a further way in which Marr's work is superior to that of most of his contemporaries. Other vision programs have usually taken no account of psychological or neurophysiological findings. In deciding what algorithm to employ, Marr makes use of both kinds of

evidence where relevant results exist. For example, having proposed a simple and elegant model of stereopsis, he abandoned it because it did not fit the psychophysical findings. He put forward a second model, which although considerably more complex is consistent with the psychophysical results. In essence it begins by matching large features in the two images and allows for the use of eye-movements to reduce disparities in matching finer detail. Again, in his account of motion perception, he suggests that Y-cells may subtract a measure of the brightness at time $t + 60$ msec from the same measure at time t : hence a cell will fire only if an edge is moving with the lighter part leading. He makes out a case that this idea is consistent with the known physiological properties of Y-cells. This is an ingenious idea for motion detection, but there is so much confusion amongst neurophysiologists about the role of X- and Y-cells that it may be premature.

Vision has two faults. First, despite Marr's emphasis on the importance of implementing algorithms as computer programs to make sure they work, it is often unclear whether he has actually written and tested a computer program to perform a particular task. Moreover, even when he claims to have implemented an algorithm, he rarely discusses how successful the program is and he gives no clear indication of the range of scenes on which it succeeds and those on which it fails. This nonchalant approach to computer modelling is unfortunately typical of the Artificial Intelligence community. Second, he is sometimes unduly dismissive of the research of others. For example, he scorns the work of Waltz and Mackworth, who wrote programs to recover depth from polyhedra, on the grounds that they were dealing with "mini-worlds". His allegation is correct, but it remains true that people can readily see depth in line-drawings of such objects and Marr provides no alternative account of how this task is performed. Both Waltz and Mackworth in fact succeeded (at different levels) in working out the constraints that enable this task to be carried out and to that extent they were amongst the few of Marr's predecessors who had a computational theory of the task they simulated.

Marr begins his preface by writing "This book is meant to be enjoyed": his intention will be fulfilled. The book is not only enjoyable, it is exciting and contains many stimulating ideas. For example, he gives good reasons for supposing that the brain cannot use iterative processes unless they converge very fast. *Vision* is perhaps the most important book on the subject to appear since Helmholtz's *Physiological Optics* was published over a hundred years ago. Marr's untimely death was a grievous loss to psychology. □

Stuart Sutherland is Director of the Centre for Research on Perception and Cognition at the University of Sussex.

NMR of biopolymers

Alfred G. Redfield

NMR in Molecular Biology. By Oleg Jardetzky and G. C. K. Roberts. Pp.681. ISBN 0-12-380580-5. (Academic: 1982.) \$59, £39.

ALTHOUGH nuclear magnetic resonance does not occupy the central role in the biological sciences that it has in analytical organic chemistry, there is hardly a branch of biochemistry or biology to which it has not been applied in recent years. The physical basis for these biological applications is the same as for organic chemistry, but the goals and general methodology differ significantly. While no unusually subtle new ideas are used, biologically relevant NMR is often complicated and controversial in its interpretation, owing to the complexity of the systems involved, and it forms a new and flourishing sub-discipline. Most current research is directed toward fine-grained local structural studies, and the determination of dynamic behaviour which only NMR can provide.

The authors of this treatise were among the innovators, and continue to be active, in this field. Their book is a thorough treatment of the most mature application of NMR to biological problems, namely the study of small molecules and macromolecules in solution, alone and in interaction with each other. Topics such as *in vivo* metabolism and NMR imaging are omitted, as is a comprehensive treatment of NMR of partly immobilized or frozen structures (commonly called "solid state NMR") except for a brief description of its application to membranes and related problems. The emphasis is therefore on proteins, especially enzymes, reflecting both the majority of current applications and the experience of the authors. Restricting the content of the book in this way was wise, since the areas not covered were in early stages of development at the time (early 1981) when the book was completed, and because the treatment of solution-state NMR could then be comprehensive in a book of reasonable length.

There are short introductory sections on fundamentals and instrumentation, and also descriptions of theories of relaxation, both paramagnetic and diamagnetic. Most



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Climate and history

Nineteen eighty two seems to be the year for books on climate and history. In addition to the two volumes reviewed by Stephen Schneider (*Nature* 298, 499) are *Climatic Change in Later Prehistory*, edited by Anthony Harding and published by Edinburgh University Press, and *Food-Climate Interactions* with Wilfrid Bach *et al.* as editors and Reidel as publisher. Both books are the records of meetings. Prices are (EUP) pbk £9.75, (Reidel) hbk Dfl. 135, \$58; pbk Dfl. 65, \$28.50.

of the book is devoted to a descriptive, often case-history, treatment of individual problems and their attempted solution. Nearly half the text covers applications to enzymes and proteins, and it concludes with chapters on nucleic acids and on membranes. There are approximately 1,500 references (out of, we are told, a possible 6,000 on the subject) and a significant number of them are discussed at least briefly.

The style is refreshingly critical, with the inevitable result that every experienced reader will find points of disagreement with certain statements or with the emphasis. For example, I felt that the treatment of nuclear spin relaxation as a tool for studies of protein dynamics overemphasizes the methods used by Professor Jardetzky and his colleagues. The novice should be

warned that nearly every topic in this field is subject to dispute but for the most part the authors do present both sides of complex issues, in references if not in their discussions. Unavoidably, certain areas are developing so rapidly that the text can provide only a useful indication of very recent advances: examples of such areas are studies of nucleic acids and of the conformation of small proteins by two-dimensional NMR.

Jardetzky and Roberts have written the most thorough treatment of the subject now available, and the first such treatise to appear in some time. Their book will be extremely useful to both experienced NMR research workers and novices alike. □

Alfred G. Redfield is Professor of Biochemistry and Physics at Brandeis University.

Energy and life

Robert M. Macnab

Biological Energy Transduction: The Uroboros. By Ronald F. Fox. Pp.279. ISBN 0-471-09026-3. (Wiley: 1982.) £24.50, \$43.80.

THE dual purpose of this book is given in its preface: first, it is intended to be an introduction, for physical scientists, to advances that have taken place in molecular biology in the past few decades; second, a description of biology from the viewpoint of energetics, suitable for senior undergraduates or graduates. These would seem to be rather distinct aims, and it is unclear why they should be pursued in a single volume — or, for that matter, why physical scientists require a special exposition of molecular biology.

An emphasis on energetic considerations in biology and their use as a unifying concept is what the title of the book leads one to expect, and, in general, the author has been successful in presenting this point of view. His style is clear and entertaining, and the major topics of interest in bioenergetics (e.g. control of metabolic pathways, storage of energy across membranes, chemi-osmosis, energetics of macromolecular synthesis) are treated well, provided one does not examine too closely the factual details presented.

In this latter regard, the book is flawed. Sometimes the error (e.g. that the bacterial flagellum is a triple helix) is incidental to the argument, but it is irritating nonetheless and one wonders how it came about. In other instances, the errors are more serious. I will cite two examples. First, on p.203, an erroneously high value for the cytoplasmic pH of bacteria is given, and then used in subsequent bioenergetic calculations. The author comments that

Many microbiologists and biochemists will be surprised to read that for bacteria grown in a medium of pH 7 the internal pH is as high as 9.

Indeed they will — did he ever ask them? (Typical experimental values are around 7.5.) Second, the choice of standard state for H₂O in conventional tabulations of free energy changes, redox potentials and so on, is incorrectly believed by the author (p.62) to be 1 M; based on this belief, he proceeds to make adjustments to 55.6 M, which of course was the standard state chosen in the original tabulations. A variety of conclusions regarding the energetics of electron transport and oxidative phosphorylation are then based on these adjusted values, and the rest of the scientific community is taken to task (p.211) for failing to make these "corrections". It is unfortunate that the author apparently did not discuss these questions with physical chemists or biochemists before codifying his viewpoint in a textbook.

The final part of the book is devoted to a

Tackling the bugaboos of solar flares

Robert Rosner

Solar Flare Magnetohydrodynamics. Edited by E.R. Priest. Pp.563. ISBN 0-677-05530-7. (Gordon & Breach: 1982.) \$89.50.

SOLAR flares have long been an enigma. Along with such problems as how the solar corona is heated, and where the "missing" solar neutrinos are, the solar flare puzzle has been a persistent reminder to astrophysicists that the gap between observation and theory is still uncomfortably large whenever the data are of sufficient quality to rigorously test the details of theoretical models.

In the case of solar flares, the difficulties may be attributed both to the classic bugaboo of theoreticians, namely phenomena in which nonlinear effects dominate (in the present case, derived from the non-linear equations of magnetohydrodynamics); and to the virtual certainty that much of the crucial physics takes place on spatial scales which are not likely to be observable in the foreseeable future (hence theory in this respect remains unconstrained by data). Given the complexity of the subject, it is welcome news that *Solar Flare Magnetohydrodynamics* does a commendable job of reviewing much of the theory relevant to present-day solar flare research.

The book is largely complementary to the recent NASA monograph on solar flares, *Proceedings of the Skylab Workshop on Solar Flares* edited by P.A. Sturrock (Colorado Associated University Press, 1980) and, if read in conjunction with this second tome, the student of solar flares will have in hand a full account of research prior to the recent flight of the Solar Maximum Mission (SMM) satellite. As such, the book should be of interest to all concerned with time-dependent

(magneto-)hydrodynamics and plasma physics in astrophysics. The material is written at a fairly advanced level, but is not so esoteric as to be inaccessible to students.

With the exception of Z. Svestka's brief chapter which reviews observations (a topic covered far better in the NASA/Skylab monograph), the book is true to its title and gives a systematic exposition of the physics of flaring plasmas, from equilibrium to possible instability, and along the way treats the associated microphysics leading to explosive heating and particle acceleration. Chapters are written by active, prominent researchers in the field, and generally convey a good sense of the current status of work in this area. Particularly noteworthy and complete are the discussions of current sheet dynamics by E. Priest, time-dependent hydrodynamics (I. Craig), evolution of post-flare plasma (G. Pneuman) and flare particle acceleration processes (J. Heyvaerts).

With the exception of J. Birn and K. Schindler's discussion of magnetic field equilibria, the chapters are well-integrated; this exceptional case, though competent in content, has a rather formal style, with virtually no attempt to mesh theory with observation.

A pleasant surprise is Priest's superb overview in the introduction, in which a precis of the entire book is given; this chapter not only ties the various contributions together, but also gives a nice perspective of the solar flare problem as a whole. The overall result is a monograph of great value; it is a must for solar physicists, and for all interested in sudden magnetic energy release and its consequences in astrophysics. □

Robert Rosner is an Assistant Professor of Astronomy at Harvard University.

Similarities in Physics

John N Shive and Robert L Weber

A new educational text stressing the similarities between apparently quite different areas of physics. The authors' unusual mission is best summarised by these extracts from their preface, addressed to the student reader:

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consideration of the evolution of the "uroboros", defined as an interlocking and at least partially self-sustaining macromolecular assembly of considerable complexity. It is suggested that, after several pre-biotic steps, an RNA polymer (generated under hygroscopic conditions) placed some selectivity on self amino-acylations; these eventually resulted, via "ur-" (= primitive) t-RNAs and ur-ribosomes, in a selectivity of linkage of the amino acids to each other. How closely the description approaches the actual process by which cells evolved is of course difficult to

ascertain, but earnest attempts such as this to construct models, which are at least plausible in terms of the properties of biomolecules, help to take the question of the origin of life out of the realm of the utterly mysterious.

Overall, I found this to be an interesting and instructive book but, in light of the factual errors it contains, it cannot be recommended as a textbook. □

Robert M. Macnab is an Associate Professor of Molecular Biophysics and Biochemistry at Yale University.

From terrestrial processes to martian features

Lionel Wilson

The Channels of Mars. By Victor R. Baker. Pp.198. US ISBN 0-292-71068-2; UK ISBN 0-85274-467-6 (University of Texas Press/Adam Hilger: 1982.) £22.50, \$39.95.

AMONG all the bodies of the Solar System yet explored in detail, Mars appears to be the only one which has had an evolutionary history sufficiently like that of the Earth to make extensive comparisons readily possible, and, at the same time, sufficiently different to make the comparisons profitable in the study of both planets.

The least-readily understood surface features of the planet are the several varieties of channel and valley showing many of the characteristics which would be associated on Earth with fluvial, glacial or permafrost processes. Present-day surface temperature and atmospheric pressure conditions on Mars preclude the existence of liquid water for other than very short periods unless large volumes of water are involved. Thus, any model of the channel-forming process which requires protracted periods of gentle water action has profound implications for the environmental conditions prevailing at the time the channels were formed. It is clearly vital to establish the duration of the channel-forming events and the times in martian history at which they occurred.

Victor Baker has been associated with the study of the martian channels since their discovery. In this welcome survey, he succeeds in presenting a generally well-balanced review of the classification systems and morphological features of the channels and of ideas on their origins, appealing widely to Earth analogues. The overall picture which emerges is one of very slow, periglacial activity alternating with brief periods of catastrophic water release. The morphological evidence for the proposed ice-related mechanisms is strong and the physical models for the processes

involving ground ice migration seem well founded.

Much of the book is devoted to those aspects of the larger channels which appear to demand catastrophic flooding by large and rapidly-released volumes of water. An extensive comparison is made between the martian features and the landforms developed in the Channeled Scabland of the north-western United States as a result of abrupt and massive water release from glacial lakes after failure of their ice dams. The morphological comparison is supported by calculation of a number of the fluid dynamic parameters associated with both martian and terrestrial floods. While the evidence for the formation of the martian channel forms by water flooding is compelling, the author is at pains to point out that we are not yet sure of the mechanisms whereby huge quantities of water are released abruptly from within the martian crust — though he does review a number of the suggestions which have appeared in print.

The style of Baker's presentation is such that it is hard to read individual chapters of this book in isolation: appreciation of the complete story requires reading the entire book. The work seems to be pitched at the undergraduate/postgraduate level, though the lay reader will have little difficulty in following the arguments and the large collection of references will be of value to the planetary science specialist. A notable feature of the book is the high quality of the many black-and-white photographs. The few typographical errors are almost all associated with numbers and equations and will be readily detected by the attentive reader. As much as anything else, the book whets one's appetite for further advances in this field. □

Lionel Wilson is a Lecturer in Planetary Sciences with the Department of Environmental Sciences, University of Lancaster.